



TAMPEREEN TEKNILLINEN YLIOPISTO
TAMPERE UNIVERSITY OF TECHNOLOGY

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CONSTRUCTION AND TESTING CRE-RECOMBINASE VECTORS
IN YEASTS *YARROWIA LIPOLYTICA* AND *PICHIA GUILLIER-*
MONDII AND EVALUATION OF DICARBOXYLIC ACID PRODUC-
TION IN β -OXIDATION BLOCKED YEASTS.

Master of Science Thesis

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ABSTRACT

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KUNDANA KANCHERLA: Construction and testing cre-recombinase vectors in yeasts *Yarrowia lipolytica* and *Pichia guilliermondii* and evaluation of dicarboxylic acid production in β -oxidation blocked yeasts.

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Dicarboxylic acids (DCAs) are derivatives of fatty acids and can be used as precursors for non-petrol-based polyesters and coatings, greases, adhesives, pharmaceuticals etc. Short chain diacids can be synthesized in high yields whereas long chain diacids production is tough and expensive because of its purification from their byproducts are in high demand. To obtain sustainable industry yeasts are considered as best example for producing diacids as they naturally produce small amount of diacids. Alkane assimilating pathway in yeast can produce DCA by using ω -oxidation pathway but the problem is that the produced diacids can be catabolized in β oxidation pathway. In the previous studies carried out at VTT the yeasts *Yarrowia lipolytica* and *Pichia guilliermondii* which were identified as promising hosts for long chain dicarboxylic acid production were modified by deleting *MFE2* gene of β -oxidation pathway. Prior these strains can be modified further for example by expressing the omega-oxidation cytochrome P450 hydroxylase complex the marker cassettes have to be removed. To this purposes Cre-recombinase/loxP recombination system was generated in this thesis.

A Cre-recombinase plasmid having Cre recombinase, Hph marker gene and autonomously replicating sequence (ARS) was constructed for *Yarrowia lipolytica*. ARS sequence cloned in this thesis work was compared with published sequences and it was similar to *Yarrowia lipolytica* ARS18 with 99.69% similarity. This shows the ARS sequence obtained in this work is equal to ARS18.

Cre-recombinase plasmid was tested in *MFE2* deleted *Yarrowia lipolytica* strain and it was able to loop out nourseothricin marker from genomic DNA. Additionally, Cre-recombinase plasmid could be looped out from *Yarrowia lipolytica* strain. Overexpression of the first enzymes of ω -oxidation- (*Nicotiana Tabacum* P450 hydrolase and *Arabidopsis thaliana* P450 reductase) in the *Yarrowia lipolytica* *MFE2* deleted strain was successful. Cultivations with 0.3 % pelagronic acid (C9 fatty acid) resulted in 78,29 mg/l of C9 diacid

production. Whereas with 1% oleic acid (C18:1 fatty acid) only substrate consumption was observed without diacid production.

With *Pichia guilliermondii* new method called Gibson assembly was used to construct the cre-recombinase plasmid. Unfortunately, only Hph marker and ARS sequence was cloned into plasmid. ARS sequence cloned in this work was compared to published sequence by using Clustal-w tool: 99.76 % similarity to the existing *P. guilliermondii* ARS sequence could be detected. Work can continue further by cloning cre-recombinase into existing plasmid and testing the plasmid in *P. guilliermondii* MFE2 deletion strains.

PREFACE

This work was done in Metabolic Engineering group at Technical Research Centre of Finland (VTT), Espoo. I would like to thank Team Leader Dr. Laura Ruohonen for giving me such a great opportunity to carry out my thesis in VTT.

I would like to thank my supervisors Dr. Kari Koivuranta and PhD Eija Rintala who helped me in encouraging and supervision. A great help in practical work and in learning new techniques at VTT. My sincere gratitude to all the people working in laboratory for helping me in turn of my thesis. Finally, I would like to thank my family members for being all the time with me.

Kundana Kancherla

LIST OF TERMS AND ABBREVIATIONS

ACS	ARS consensus sequences
ADP	adenosine Di phosphate
ARS	Autonomously replicating sequences
ATP	Adenosine Triphosphate
β oxidation	Beta Oxidation
CEN	Centromere
Cre	Cre recombinase plasmid
DCA	Dicarboxylic acid
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
FAA	Fatty acid Activation
FAD	Flavin Adenine Dinucleotide
FADH ₂	reduced Flavin Adenine Dinucleotide
FAO	Fatty alcohol oxidase
H ₂ O ₂	Hydrogen Peroxide
HCL	Hydrogen Chloride
Hph	Hygromycin Marker
MgCl ₂	Magnesium Chloride
NAD ⁺	Nicotinamide Adenine dinucleotide
NADH	reduced Nicotinamide adenine dinucleotide
ORI	Origin of Replication
ω	Omega
<i>P.guilliermondii</i>	<i>Pichia guilliermondi</i>
<i>S.cerviciae</i>	<i>Saccharomyces cerevisiae</i>
SDS	Sodium Dodecyl Sulphate Gel Electrophoresis
SSB	Single strand DNA binding proteins
<i>Y.lipolytica</i>	<i>Yarrowia lipolytica</i>

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1. INTRODUCTION

Development of new applications from oils and fats were the important criteria to obtain sustainable industry. One application was to use fatty acid-derived products like dicarboxylic acids as they were considered as building blocks of polymer (Huf et.al, 2011). ω -hydroxy fatty acids were valuable compounds in chemical industry and were raw materials for industrial sectors (Bitto et.al, 2009).

Bioplastics were the polymers that were derived from renewable biomass and they were biodegradable. Production of polyamides and polyester synthesis were mainly processed via chemical synthesis. This production of polyamide and polyester through fats and oils was a novel technology in industrial field. However, there had not been much development in strains to produce di-carboxylic acids and no huge scale of manufacturing process. (Huf et.al, 2011) There were also many uses with other diacids like the lithium salts of C9 acid were used as lubricants and also used as additives to antifreeze mixtures. Azelaic acid itself had more number of antibiotic properties, which were used in treatment of skin. (Green et.al, 2000)

Yarrowia lipolytica and *Pichia guilliermondii* are recognized as promising hosts for long chain dicarboxylic acid production. These yeasts can use long chain alkanes and fatty acids as the sole carbon sources. Degradation of alkanes and fatty acid metabolic pathways were studied intensively in above-mentioned yeasts. Metabolism of long chain fatty acids and alkanes contains import of substrates and sequential degradation with two pathways: ω -oxidation pathway and β -oxidation pathway. They occur in two different locations: ω -oxidation pathway occurs in endoplasmic reticulum and β -oxidation pathway takes place in peroxisomes. DCA production in yeast can be enhanced by genetic modifications e.g. by blocking β -oxidation and by enhancing ω -oxidation. (Cheng, et. al, 2005).

Cre recombinase-loxP system uses a site-specific recombinase Cre from bacteriophage P1 that catalyses recombination between DNA recognition sites called loxP. This site had 34bp consensus sequences by two 13bp-inverted repeats, which are separated by 8bp core sequence (Ribeiro et.al, 2007). This is a very efficient method for marker removal from recombinant yeast strains. This system is widely used both in prokaryotic and eukaryotic organisms. The excision leaves behind a single loxP site.

There were no commercial vectors available for *Yarrowia lipolytica* and *Pichia guilliermondii*. Autonomously replicating sequences based vectors were described for these strains so ARS sequence are used as starting point for constructing these plasmid vectors.

In this work a new technique called Gibson assembly had been used, this technique were developed by Dr.Daniel Gibson at J.Craig Venter Institute and this method had been licensed to New England's BioLabs Inc., This technique is used mainly in assembling different length DNA fragments. This technique was very easy to use and efficiently joins multiple overlapping DNA fragments in a single tube. The main principle of this Gibson master mix is that exonuclease creates a single stranded 3' overhang which aids the annealing of fragments, polymerase fills the gaps and DNA ligase nicks the assembled fragment. (Gibson, 2011, New England Biolabs, Inc.,)

The aim of this work was to construct plasmid vectors with codon optimised Cre-recombinase genes and to test the constructed Cre-recombinase vectors by removing antibiotic marker from *MFE2* deletion strains.

2. LITERATURE REVIEW

2.1 Dicarboxylic acids

α - ω dicarboxylic acids were widely used as raw materials for synthesizing plastics, polymers, adhesives, perfumes and lubricants with high quality (Liu, et. al, 2004). The majority of industrial dicarboxylic acids were produced through chemical conversion. Shorter chain DCAs were manufactured by Pd (II) catalyst with high pressure and temperature. Due to many applications of DCA products with long chain carbon length, costs were also increased as it was very expensive process. There are many companies involved in industrial production of long chain DCA by *Candida tropicalis* and to development of fermentation processes. Because of high demand in removal of byproducts from long chain DCA products, purification was more difficult and more cost effective process. (Huf, et.al, 2011, Kogure, et.al, 2007).

Long chain α , ω -dicarboxylic acids had been mainly produced by microbial fermentation or oxidation. In 1980's yeasts, *Candida tropicalis* and *Candida cloacae* were used to transform n-alkanes and fatty acids to dicarboxylic acids. However, there are many environmental problems raised due to commercial production of long chain α , ω -dicarboxylic acids. (Ngo, et.al, 2006).

2.2 Beta-oxidation pathway

Fatty acids were important molecules for life of a cell and they produce high yield of ATP, which acts as a fuel for cell, upon degradation in β oxidation pathway (Hettema, and Tabak 2000). The ability to take fatty acids or its derivatives up from environment was important when there is not available other nutrients. In presence of carbon source, the ability of cells to import fatty acids was advantageous as they can use existing molecules than needlessly consuming energy required for biosynthesis. Fatty acid uptake mechanism in eukaryotes was not well understood but there was an evidence that fatty acid transport was protein dependent and saturable process for long chain fatty acids and this proposed other model in prokaryotes that membrane bound transporter protein was responsible for uptake of exogenous fatty acids. In yeast, the fatty acid metabolism was confined to single organelle that was peroxisomal β -oxidation process that helped to turn out yeast into an attractive model organism to study about degradation of fatty acids. (Hiltunen, et.al, 2003)

In yeast cellular fatty acid was fed by long chain fatty acyl CoA that were formed by two enzyme systems: fatty acid synthetase (FAS) and acetyl-CoA carboxylase (Acc1p). Secondly mobilisation of stored fatty acids from lipids. Thirdly uptake of

exogenous fatty acids. The synthesis of free fatty acids yields acyl-CoA esters which were further activated by mobilisation. These acyl-CoA esters can be utilised in lipid synthesis, protein acylation or fatty acid permeation. Two different classes of proteins were observed to stimulate the uptake of fatty acids. First class proteins contains fatty acids with high affinity and acts as both intracellular or extracellular fatty acid receptors. The other class of proteins are acyl Co-A synthetases which severely effects the uptake of fatty acid. A novel protein named fatty acid transport protein (FATP) and fatty acyl CoA synthetases (FACS) increased the uptake of long chain fatty acid. $\Delta fat1p$ cells in a fatty acid metabolising process resulted decrease of uptake fatty acid. (Hettema, and Tabak, 2000)

Long chain fatty acids were transported as CoA esters into peroxisomes through peroxisomal membrane via Pxa1p and Pxa2p (Pat1p and Pat2p) transporters that belong to ABC transporters (Hiltunen, et. al, 2003). The mechanism of fatty acid transport across the membrane by transporters Pxa1p-Pxa2p is unclear but it is premised that CoA polar group activated fatty acid was transferred across the peroxisomal membrane by Pxa1p-Pxa2p in ATP dependent manner (Hettema and Tabak, 2000).

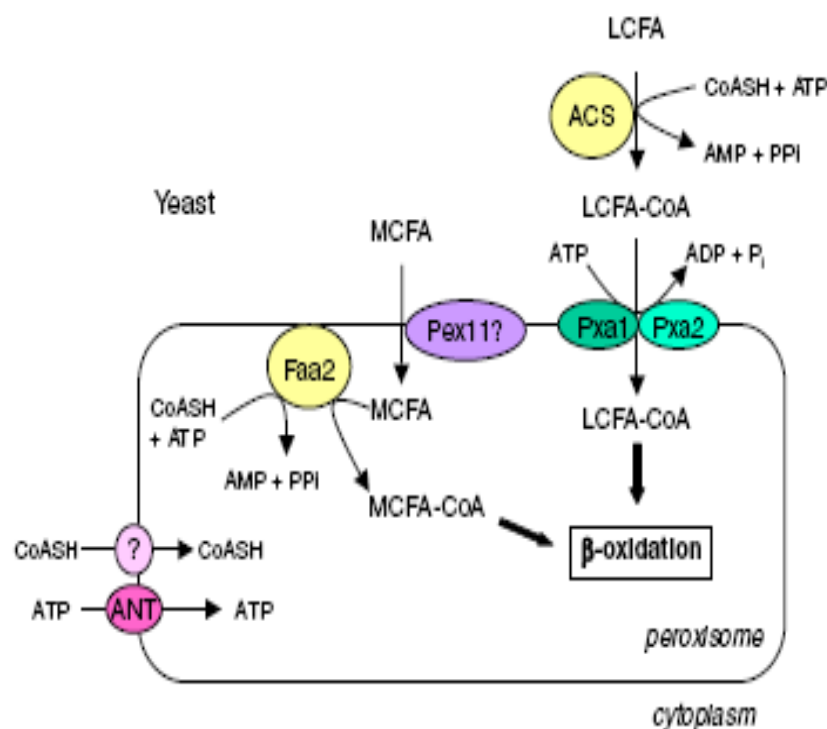


Figure 1: The above figure explains functions of ANT and PXAp (Theodouloua, et. al, 2006).

According to Hettema et.al, (1996) medium chain fatty acids enter peroxisomes as free fatty acids, which were then activated by fatty acid activator Faa2p. In his study- in $\Delta faa2p$ strain β -oxidation of medium chain fatty acids was destroyed. These shows that *faa2p* has a role in the activation of MCFA's. But there was still an unclear mechanism

that how MCFAs cross the peroxisomal membrane itself ?It can be either by passive flip flop from cytoplasmic leaflet of membrane or by protein-mediated process.

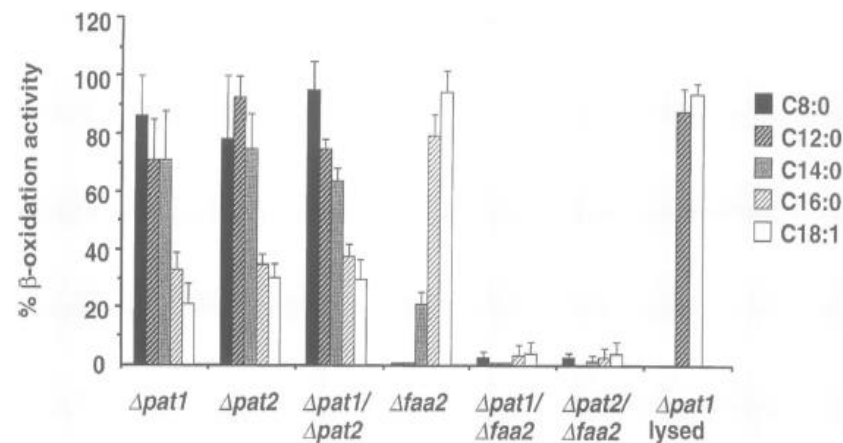


Figure 2. The above figure shows the two separate pathways that are involved in the degradation of LCFAs and MCFAs in peroxisomal fatty acid β oxidation. (Hettema, and Tabak, 1996)

Peroxisomal beta-oxidation pathway contains four steps: 1. Dehydration 2. Hydration 3. Dehydration and 4. Thiolitic cleavage (Poian et.al, 2012). Fatty acids were catabolised and 2 carbons shortened acyl Co-A and acetyl CoA were formed. Fatty acid activation to CoA-esters was essential to enter into beta-oxidation pathway (see below). (Roermond, et. al, 2003)

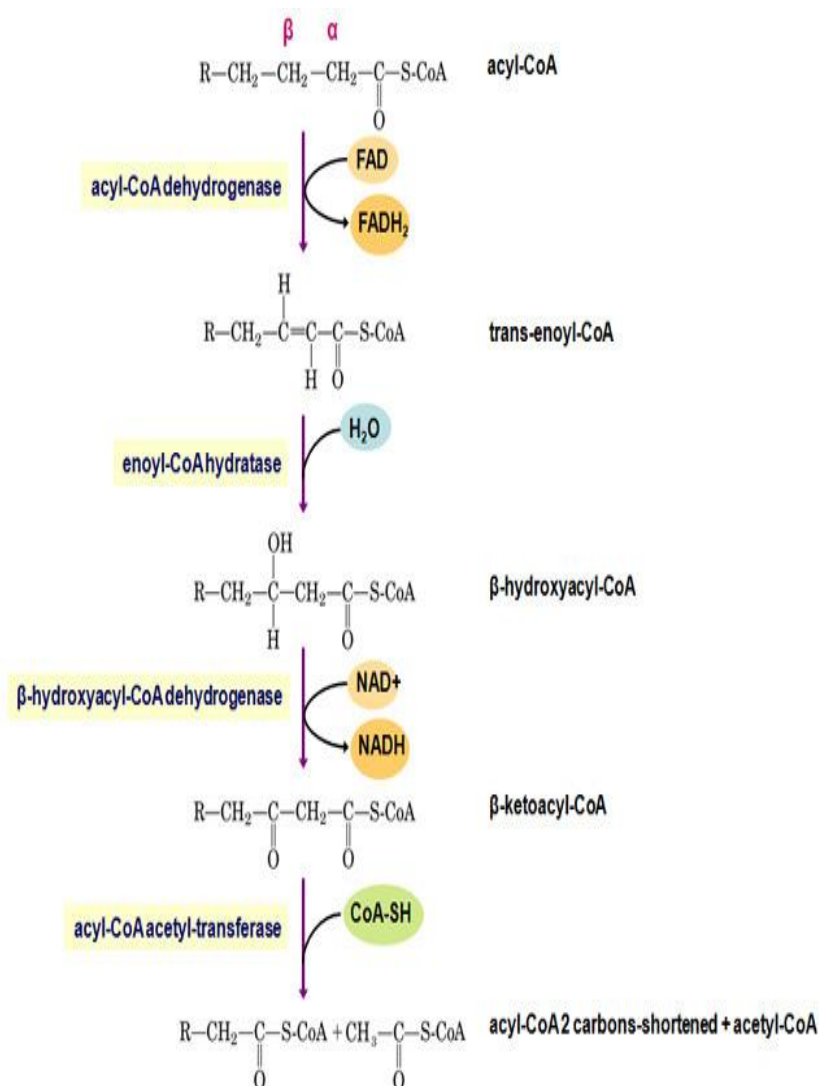


Figure 3. Reactions involved in beta-oxidation pathway in peroxisomes. (Poian, et.al, 2010).

The enzymes involved in beta-oxidation were Pox1p/Fox1p, Mfe2p/Fox2p and Pot1p/Fox3p. The β -oxidation process starts with oxidation of acyl-CoA substrate into trans-enoyl-CoA by FAD dependent enzymes acyl-CoA oxidase Pox1p/Fox1p. Pox1p/Fox1p passes the electrons directly to oxygen to generate H₂O₂ (Hiltunen, et.al, 2003). In *Yarrowia lipolytica* there are several acyl-CoA oxidase isoenzymes with different chain length specificity (Wang, et.al., 1999(a)).

2.2.1 Peroxisomal multifunctional enzyme type 2

The physiological activity of this enzyme had been identified from *Saccharomyces cerevisiae*. This enzyme catalyses the dehydrogenation and hydration of trans-2-enoyl-CoA substrate through an R-specific pathway (Ylianttila, M. 2005). MFE-2 type proteins mainly present in yeast had three functional domains with one polypeptide chain (Poirier, et.al, 2006).

Multifunctional enzymes type 1 and multifunctional enzyme type 2 contain 3-hydroxyacyl-CoA dehydrogenase and 2-enoyl-CoA hydratase activities. The main difference between them is that Type 1 is specific to S-isomeric substrates and type 2 was specific to R-isomers. The 3-hydroxyacyl-CoA dehydrogenase of MFE2 had a very interesting feature; it undergoes duplication and obtains two 3R-hydroxyacyl-CoA dehydrogenases in a same polypeptide chain but with different specificities of chain length. The first dehydrogenase A in amino terminus catalyses the reaction for long and medium chain substrates that are like 3R-OH-C10-(3R)-OH-C16. The second dehydrogenase B region sequence between hydratase 2 and dehydrogenase A in MFE-2 shows the activity between 3R-OH-C4. (Ylianttila, et al., 2006).

Mfe2p/Fox2p dehydrogenase enzymes in mammals had been duplicated domain organization Domain A and Domain B, Domain B had highest activity towards short chain substrates and Domain A had activity towards long chain fatty acids. Inactivation of any domains introducing point mutations into DNA sites shows them both are active enzymatically (Qin, et.al, 1999, Hiltunen and Qin. 2000). The yeast hydratase enzymes and mammalian enzymes were related to each other in amino acid sequence but they were different in their kinetic activities (Jiang, et.al, 1996).

The last step in beta-oxidation is carried out with ketoacyl-CoA which undergoes thiolytic cleavage by Pot1p/Fox3p enzymes and produces acetyl-CoA and a 2 carbons shortened AcylCoA (Einerhand, et.al, 1991).

2.3 ω (omega)-oxidation pathway

Verkade and his colleagues discovered ω -Oxidation at 1932 in Netherlands by feeding fatty acids with different chain lengths to dogs and they observed these resulting urinary dicarboxylic acids (Coon, 2005).

In omega-oxidation the fatty acid was converted into dicarboxylic acid by three-step pathway (Eirich, et. al, 2004). In the first step fatty acids were catalyzed into ω -hydroxy fatty acids by cytochrome P450 monooxygenase and a NADPH: cytochrome P450 oxidoreductase complex. (Eschenfeldt, et.al, 2003). According to Scheller, et.al, 1998 the first step in ω -oxidation pathway is only considered sure and it is performed by hydroxylase complex present in endoplasmic reticulum membrane and forms by P450 monooxygenases and NADH dependent P450 reductase. ALK genes that belong to CYP52 family code these monooxygenases. It had proved in *Candida maltosa* by overexpressing P-450 52A3. This strain had been able to perform all steps in omega oxidation to produce DCA. (Scheller, et.al, 1998)

In second step, the alcohol produced in the first step is further oxidized to an aldehyde by fatty alcohol oxidase (FAO). Finally, dicarboxylic acids were produced from fatty acid aldehyde by fatty aldehyde dehydrogenase (FAHdH). (Eirich, et.al, 2004)

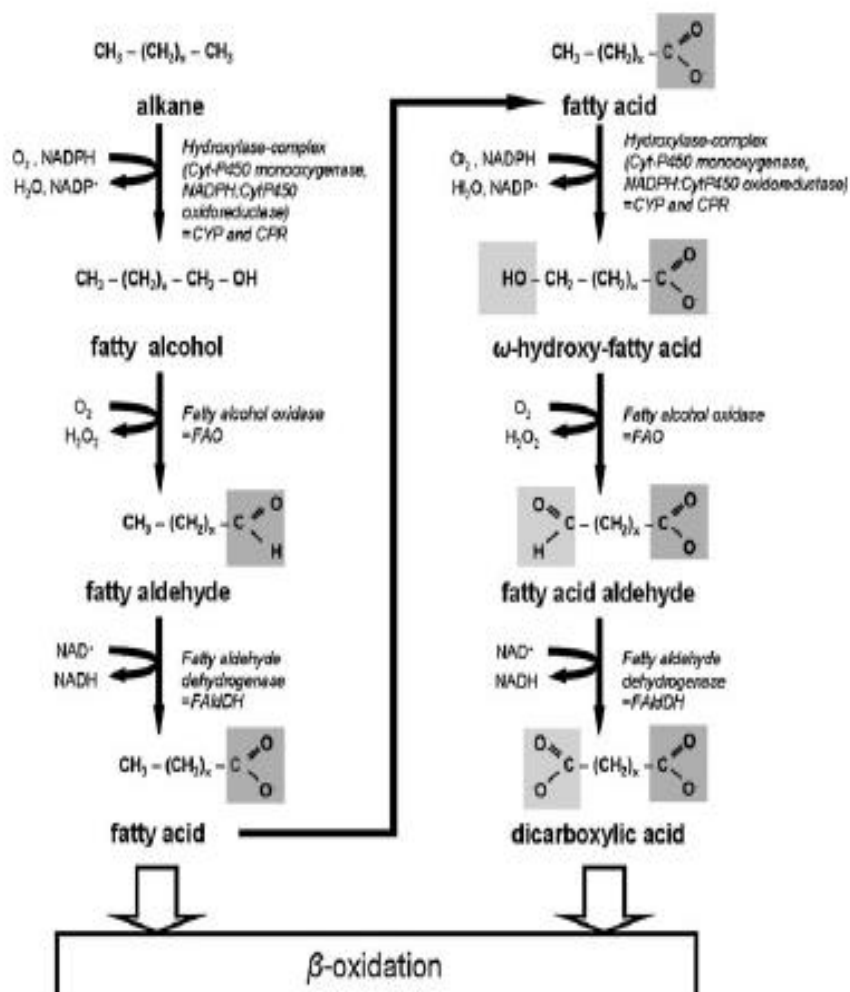


Figure 4. The above figure explains the terminal oxidation of alkanes and fatty acids. Diacids were produced through omega oxidation, which was used in alkane utilisation and takes place in Endoplasmic Reticulum. Whereas beta oxidation holds in peroxisomes (Huf, et. al, .2011).

According to Eirich, et.al, 2004 *Candida tropicalis* strain ATCC 20336 can convert fatty acids into long chain dicarboxylic acids via ω -hydroxy fatty and ω -aldehydes by a fatty alcohol oxidase gene (FAO). In this strain β oxidation is blocked and three FAO genes have been cloned (FAO1 and FAO2a and FAO2b). Fermentations were performed by using High oleic sunflower fatty acid (HOSFFA) as a substrate and glucose as a cosubstrate. There was no FAO activity for 2 to 4 hours but a rapid increase of peak had been seen after 30 to 40 hours of post induction of HOSFFA substrate. DNA sequences of clones were compared to previously cloned fatty alcohol oxidase (FAOT) and it has been concluded that FAOT is different from FAO's sequences. Relative activities of FAO1, FAO2a and FAO2b on 1-alkanols and 2-alkanols were studied by using 20mM acetone concentration.

An interesting result observed that FAO1 utilizes omega-hydroxy fatty acids as substrate and FAO2 utilizes 2-alkanols as substrate. This concludes that FAO1 is more expressed in fatty acid conversion to diacids. (Eirich, et.al, 2004)

2.4 Cytochrome P-450 Monooxygenases (P450s, CYPs)

In eukaryotes cytochrome P450s play a key role in several reactions especially in steroid biosynthesis that takes place either in endoplasmic reticulum or in mitochondria (Makovec and Breskvar.1998). Cytochrome P450 monooxygenases were comprised of a large family enzymes which function as enzyme catalysts and were mainly involved in synthesis of steroids, lipids, vitamins, sterols etc., they were also involved in xenobiotic, carcinogens, biotransformation of drugs and some biosynthesis of natural products(Kurtzman, et.al.,2011). There were 21 sequences presently belonging to CYP52 family under the P450 nomenclature super family, which involved in terminal oxidation of long chain n-alkanes. Which was also the rate determining step of n-alkane degradation pathway (Scheller, et.al,1998).

P450_{BM3} was a best example for redox partner enzymes where P450 enzymes were fused to generate multidomain entities. P450_{BM3} enzymes were flavocytochrome enzymes formed by fusion of soluble, fatty acid hydroxylase P450 to cytochrome P450 reductase. These enzymes oxygenate ω 1- ω 3 carbons with saturated fatty acids of chain length carbon C₁₂ – C₁₈ and different polyunsaturated and saturated fatty acids. (Munro et.al, 2007)

2.4.1 CYP52 Family

The Cytochrome P52 (CYP52,P450 Alk) family was composed of many subfamilies, mainly they are found in n-alkane assimilating *Candida* species like *Candida tropicalis*, *Candida lipolytica*, *Candida bombicola* and *Candida maltosa*. The Alk enzymes were responsible for initial and rate-limiting step of hydroxylation of fatty acids and alkanes. These products were oxidized further and finally metabolized through β -oxidation pathway. Numerous CYP52 genes were involved in encoding isozymes, which have different overlapping substrate specificities. These genes were mostly inducible by exogenous like alkenes, n-alkanes, fatty acids and fatty alcohols of different chain length (Cresnar and Petric.2010). The rate-limiting step in conversion of n-alkanes to fatty acids and then to aliphatic diacids were carried out by CYP52 protein and hence this CYP52 gene was necessary to overexpress to enhance DCA production (Craft et.al, 2003).

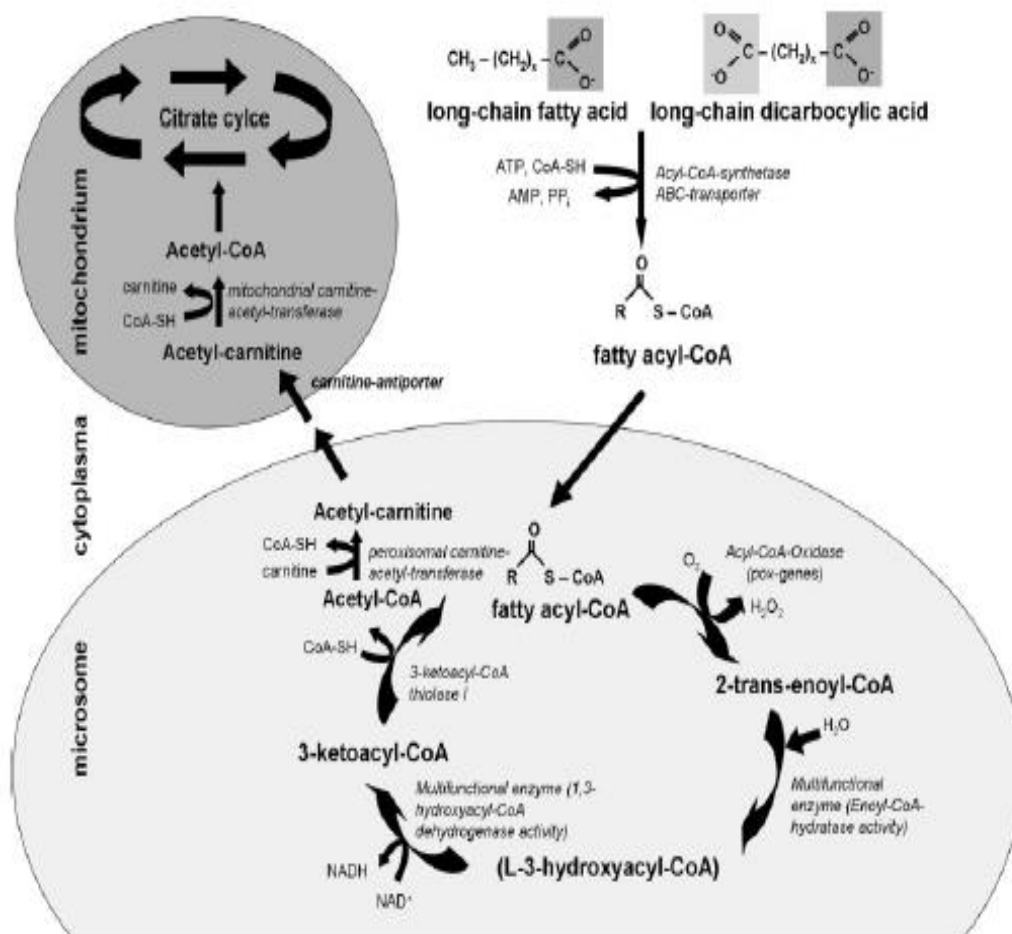


Figure: 5. In this pathway it showed that α and ω diacids transportation to peroxisomes in the form of fatty acyl-CoA and then β -oxidation pathway in peroxisomes. Product of beta-oxidation acetyl-CoA is transferred into mitochondria by carnitine-antiporter and became a starting product of citric acid cycle (Huf, et. al, 2011).

2.5 ARS

In yeast genome, there are short specific DNA sequences, with A and T rich base pairs know as autonomously replicating sequences. These sequences have unique ability of high frequency transformation and maintain stable plasmid. (Dhar, et.al, 2012)

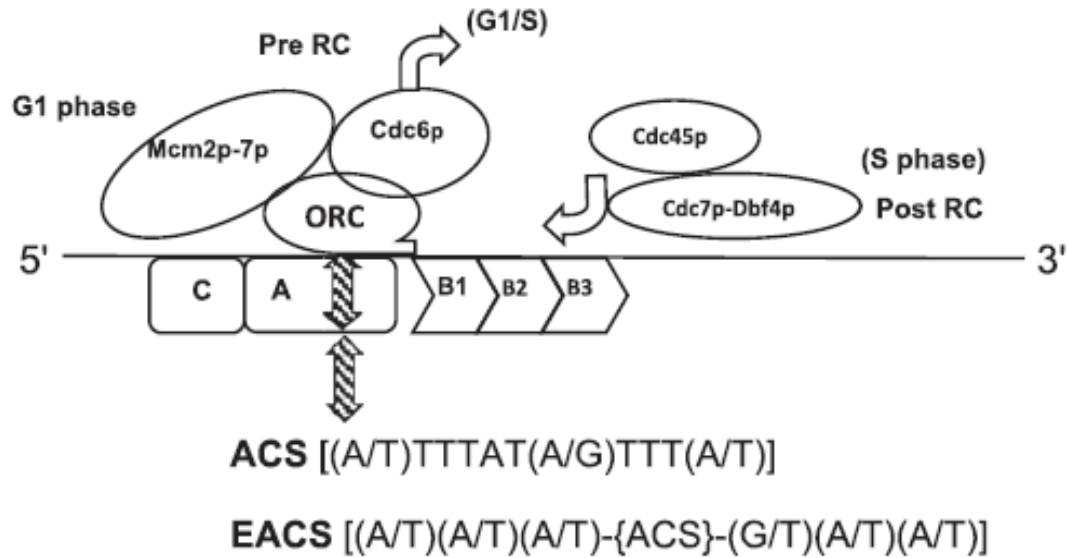


Figure 6. In this figure it explained clearly about the structure of autonomously replicating sequences with different domains Domain A, Domain B (B1, B2, and B3) and Domain C (Dhar, et.al, 2012).

2.5.1 Domain A

Domain A had a sequence of 11 base pairs called as ARS consensus sequence (ACS) [(A/T) TTTAT (A/G) TTT (A/T)]. In most of the eukaryotes, the above sequence was comprised with origin recognition complex (ORC) as this was a protein initiator in cells. (Chang, et al., 2008). ORC main function was its ability to bind DNA origin and ACS sequence was necessary to bind ORC-DNA binding and origin function. B elements enhance the function and ORC protects 50bp of DNA that contains B1 and ACS sequences. Binding of these two regions together known as ORC-DNA binding and as a core region of origin function (Lee and Bell 1997). ACS element comprises of A-T rich consensus sequence and an active ARS contains 9/11 or 10/11 match sequences. ACS binds to ORC Origin Recognition Complex and necessary for initiation of replication. (Dhar, et.al, 2012)

2.5.2 Domain B

Domain B, was for the function of ARS, a 100 base pairs *cis* acting element located to downstream from ACS. A mutation in this domain reduced the serenity of DNA unwinding and capability of replication origin, hence, these were known as DUEs (DNA unwinding elements) and no consensus sequences were found in this domain. Domain B consists of short sequences in its downstream and was divided in B1, B2, B3 and B4 elements. Element B1 had high affinity to ORC binding and its function as replicator. An ORC contact with DNA sequence elements and contains few elements from B1 that activates replication origins in S phase. Element B2 interacts with Single strand DNA binding

(SSB) proteins in DNA. Element B3 binds to ARS binding factor 1, this helps for cell viability in yeast and activates transcription, triggers DNA replication and silencing the gene. Element B4 had a capability of substituting remaining B elements. (Dhar, et al., 2012).

2.5.3 Domain C

Domain C consists of 200 base pairs extends to the left side of domain A. Plasmids were destabilized when there were deletions in elements Domain B and Domain C but it does not terminate replication as it happens in Domain A (Celniker et al., 1984).

2.5.4 ARS in *Yarrowia lipolytica*

ORI – (Origin of Replication) alone cannot maintain plasmid extrachromosomally, centromeric sequence was compulsory to maintain a stable plasmid. Totally, three ARS had been identified for *Y. lipolytica*. All of them carry a centromere sequence and chromosomal origin of replication: ORI3018/CEN3, ORI4002/CEN4 and ORI1068/CEN1. All the sequences were able to induce LEU2 locus and CEN 1 & 3 were closely related to chromosomal centromere, it also corresponds to pause the polymerase site. Both CEN and ORI functions individual regions of ARS and can exchange between ARS and CEN. Thus chromosomal ORI was required for *Yarrowia lipolytica* ARS. But only two of ARS sequences had been confirmed: ARS18 and ARS 68. All the sequences were able to induce chromosome breakage when they were integrated to *LEU2* locus. ORI and Centromere functions were carried individually by ARS inserts (Barth and Gaillardin 1997).

In genome replication, origins were distributed for every 20 kb to 50 kb and chromosomal origins near to centromere were needed for autonomous replication. Thus a CEN sequence was always needed for replication in *Yarrowia lipolytica* (Vernis et al., 1998). *Yarrowia lipolytica* CEN derived sequences do not have indicative sequence similarity but share more DNA structural motifs which include juxtaposition of conserved repeats and few contain dyad symmetries. The genome sequence of *Yarrowia lipolytica* centromeres had similar features present in higher eukaryotes and fungi (Vernis, 2001). ARS18 and ARS68 sequences of *Yarrowia lipolytica* were identified as supporting extra chromosomal replication with each carrying 1-kb of centromere (CEN). Plasmids with these ARS sequences transform with high rate of frequency and were more stable than any other ARS plasmids (Fournier, et al., 1993).

2.6 *Yarrowia lipolytica*

Yarrowia lipolytica was an ascomycetous yeast and originally classified as *Candida lipolytica*. In middle of sixties, it has been recognized as important industrial interest as it was able to use n-paraffin as a sole carbon source. These were non-conventional yeast species and can be isolated from dairy products. It mainly used glucose, acetate, alcohols

and hydrophobic substrates like alkanes and fatty acids as a carbon sources. It had 20.5Mb of genome, which contains with 49% of G+C content and made up of six chromosomes (Barth and Gaillardin 1997).

Yarrowia lipolytica was dimorphic yeast because it either can be as a true mycelium or yeast-like cells based on available conditions (Casaregola et. al., 2000). They can be exhibit in many colony shapes glistening and smoothy kind to heavily convoluted form. These strains are strictly aerobic strains and most of them cannot grow above 32°C. Hence they were not considered as pathogenic strain. Most important factor was it can associate with hydrophobic substrates or protenaceous. (Thevenieau, et.al, 2009).

In *Yarrowia lipolytica* there were two long chain fatty acid synthetases: Acyl-CoA synthetase-I that was phosphatidylcholine independent and involves in cellular lipid synthesis. Acyl-CoA synthetase-II was phosphatidylcholine dependent and produces acyl-CoA which was degraded by β -oxidation (Mishina, et.al., 1978). This peroxisomal ACS II requires ATP provided by Adenine nucleotide transporter protein (Ant1p). In *S. cerevisiae* Ant1p was an integral protein of peroxisomal membrane and it was inducible by oleic acids. (Roermund et. al, 2003, Palmier, et.al, 2001)

POX genes were identified as genes that encode acyl-CoA oxidase in *Yarrowia lipolytica*. There were five POX genes identified in this *Y. lipolytica* whereas there exists only one gene in *S. cerevisiae* and three in *Candida tropicalis*. First step of β -oxidation pathway is catalyzed by Acyl-CoA oxidases, that was the oxidation of acyl-CoA to trans-enoyl-CoA. The physiological functions of these oxidase enzymes was studied by disrupting genes. Acyl Coenzyme A Oxidase (Aox) isozyme activities were determined by using oleic acid and glucose as a substrate. Single null mutations did not affect much to growth but it affected the acyl-CoA oxidase activity. This had been the first POX disrupted gene in *Yarrowia lipolytica*. MTLY20 construct was $\Delta POX2$ and $\Delta POX3$, there were a defect on oleic growth plates and there were no effect with liquid YNO medium. There was no growth observed in MTLY37 construct and in this construct four POX genes were deleted $\Delta POX2$, $\Delta POX3$, $\Delta POX4$ and $\Delta POX5$ genes. After comparing all the combination of disruption POX genes it was concluded that POX2 and POX3 codes specific chain length of Aox, POX4 codes for small Aox partial growth and POX5 codes for non-specific chain length. Aox2 were more active against long chain fatty acids and Aox3 show active towards short chain fatty acids. This explains that lack of Aox activity and no growth on oleic acid can be used to study in vivo import of Aox genes into peroxisomes. *Yarrowia lipolytica* had Aox isoenzymes that were more complex than in other yeasts and these show its ability to grow on hydrophobic substrates like fatty acids, fat and alkanes. (Wang, et. al, 1999(b))

2.7 *Candida tropicalis*

Long chain dicarboxylic acids in *Candida tropicalis* are produced by aerobic and viscous fermentation system. During this process oxygen supply had an important role. Lack of this supply leads to the suboptimal productivities and low quality products were produced. Therefore, limitation of oxygen supply in this system was a major problem for the production. Modified reactor designs, using oxygen vectors or using pure oxygen through gas inlet are few techniques, which had not been successful. A new method had introduced to overcome the problem, namely, gas-liquid transport system. This method is not only to increase oxygen supply but also enhance the rate of metabolism. CT1-12 strain *Candida tropicalis* showed that adding H_2O_2 to the fermentation system could be converted to oxygen, which is available directly from culture and water by a catalase enzyme. It also increases the metabolism of cytochrome P450. High concentration of H_2O_2 is toxic to cells but low concentration increases the oxygen supply. The DCA production of brassylic acids increased from 16.5g/l to 22.7g/l approximately in 80hour 2mM H_2O_2 concentration. (Jiao, et.al, 2001)

There was a search for best yeast producer of sebatic fatty acid and brassylic acid. Different cultures were grown using n-alkanes like decane and tridecane as a sole carbon source with strains like *Saccharomyces*, *Torulopsis*, *Hansenula*, *Yarrowia*, *Pichia* and *Candida*. Out of 200 different cultures *Candida tropicalis* Tv-8 strain was choosed as a best strain for producing highest amount of Sebatic acid when decane was used as a carbon source. Tv-8 *Candida tropicalis* produced 4.2mg/100ml where as other strains produced ≤ 3 mg/100ml of medium. (Ulezlo and Rogozin. 2003)

2.8 *Pichia guilliermondii*

Pichia guilliermondii, is classified as an asporogenous species, were formerly called as *Candida guilliermondii*. This species were recognised as industrial interest yeast because of its flavinogenic nature. *P. guilliermondii* is considered as model organism because of its high potential of converting xylose to xylitol; anti caries, sweetner. Genetics of this yeast species were studied primarily based on its ability to use hydrocarbons (n-hexadecane) as sole carbon and energy sources. It is capable of producing single cell protein hydrocarbons and utilizes hemi cellulosic hydrolysates obtained from acid hydrolysis. Ability of overproducing riboflavin during lack of iron deficient medium was a special feature of this strain. (Sibirny, et.al, 2009 and Boretsky, et.al.,1999)

Pichia guilliermondii ARS was called as PgARS and it is located at 3' end of the RIBI open reading frame. This strain had similar ARS features like other yeasts with 71% of A+T base pairs content. PgARS were used to develop a high efficient transformation system. Optimal temperature for this strain is 30 °C and had an upper limit of 42 °C. Construction of knockout strains, selection markers, as well as ARS elements provides a

promising tool for developing molecular biology technical application interest in industrial atmosphere. (Sibirny, et.al, 2009 and Boretsky, et.al.,1999)

2.9 Industry

The market value of dicarboxylic acids was very high and there were few companies, which were into market for production.

Casda Biomaterials Co., Ltd.

This plant had a history of 24years and it was a largest plant in china for producing sebacic acid. It produces 23000 tons of per annum and mainly used in nylon industry. Because of its quality, it reached too many countries like US, Europe (Italy, Holland, Belgium and France) and Japan.Casda Biomaterials, China.2013.

Cathay Industrial Biotech

This company started just over 10years ago in china and had a huge commercial scale production of long chain dicarboxylic acids. It currently produces dibasic acids from C11-C16 fatty acids.Cathay Industrial Biotech, China.2013.

Sebacic India Limited

This was a huge plant and first Company in India to produce Sebacic Acid which worth Rs.93 Crores with 10000 Metric Ton Per Annum of Sebacic acid. This company also produces Mixed Fatty Acid, Glycerin, 2-Octanol and Sodium Sulphate. Sebacic India Limited, India.2013.

Verdezyne-Green chemistry

This company produce the intermediates of plastics and nylon by using yeast fermentation technology. They have set up a platform where yeast can utilize a variety of plant-based oils and by-products of them. Dodecanedioic acid (C12) mainly used for lubricant and adhesives were traditionally produced from butadiene by chemical process but by using Yeast technology cost effective Dodecanedioic acid is produced. Total capacity of this plant is Adipic acid-6.3\$ billion per annum, Sebacic acid- 600\$ million per annum and Dodecanedioic acid- 250\$ million per annum was produced.Verdezyne, USA.2013.

Zibo Guangtong Chemical Co., Ltd

A sub company from Zibo named Diacid plant had been started recently 1997 by Chinese Academy of Science. This plant produces long chain dicarboxylic acids by using biologic zymolysis methods. It produces C12, C13, C14, C15 diacids, dinitrile and diamine. This

company has ISO9001 certification and got many awards globally. Zibo Guangtong Chemical Co., Limited, China.2013.

Other Companies involved in diacids production

- BioAmber, USA.2013.
- Itaconix, USA.2013.
- Myriant, USA.2013.

3. AIM

- 1) To construct plasmid vectors containing codon-optimised Cre-recombinase and marker genes with organism- specific promoters and terminators for *Yarrowia lipolytica* and *Pichia guilliermondii*
- 2.) To test the constructed Cre-recombinase vectors by removing the antibiotic marker from strains where a gene encoding enzymes of β -oxidation (*MFE2*) had been deleted.
- 3.) To overexpress the first enzymes of ω -oxidation (P450 hydroxylase and reductase) in the strains where β -oxidation is blocked.
- 4.) To test the dicarboxylic acid production of the constructed strains.

4. MATERIALS AND METHODS

4.1 Yeast and bacterial strains

Two different yeast strains had been used in this work: The first strain is *Yarrowia lipolytica*C-00365 and the second yeast strain is *Pichia guilliermondii*C-72064 from VTT culture collection. According to VTT studies both of them were capable to alkane utilization.

Bacterial transformations had been carried with *E. coli* electrocompetent TOP10 (Invitrogen, USA) and DH5 α (F⁻, endA1, recA1, hsdR17, gyrA96, relA1, ϕ 80d Δ lacZM15) strains.

4.2 Media and growth conditions

4.2.1 Culture medium

The standard YPD medium has 2 % Bacto-peptone (BD, USA), 1% Bacto-yeast extract (BD, USA) and 2 % of Glucose (Sigma-Aldrich, Germany).

Bacterial cultivations had been carried out in LB medium containing 1 % Bacto-tryptone (BD, USA), 0.5 % Bacto-yeast extract (BD, USA) and 1% NaCl. The plasmid selection was carried by adding 100 μ g/ml Ampicillin. LB agar plates were prepared by adding 1.5 % Bacto-agar (BD, USA).

4.2.2 TE buffer

TE buffer contains 10 mM Tris-HCl and 1 mM EDTA at pH 8.0.

4.2.3 STET buffer

Stet buffer contains 8% sucrose, 50mM Tris-HCL, pH 8.0, 50mM EDTA, 5% Triton X-100.

4.3 Genomic DNA isolation

C-00365 and C-72064, which were grown on 10 ml of YPD overnight at +30°C in 250 rpm, were centrifuged for 5 minutes with 3000 rpm. Supernatant had been decanted and the pellet had been resuspended with 1 ml of sterile double distilled water and transferred into a new 1.5 ml tube. After 30 seconds centrifugation, supernatant was removed and pellet had been resuspended in residual liquid (10 μ l). 0.2 ml of plasmid release solution (2% Triton X-100, 1 % SDS, 100 mM NaCl, 10 mM Tris-HCL (pH 8), 1 mM Na₂ EDTA) and

0.1 ml of phenol, 0.1 ml of chloroform:isoamyl alcohol (24:1) were added. After 0.3 grams of acid-washed glass beads was added, tubes were vortexed for 4 minutes. 0.2 ml of TE (pH 8) had been added and samples were centrifuged for 5 minutes at 13500 rpm. The upper (water) phase was transferred into a new tube. The phenol: chloroform: isoamyl alcohol step were repeated twice to increase the concentration of DNA. Upper phase was transferred into a new tube and 1 ml of cold 94% EtOH were added and tube was mixed by inverting, centrifuged for 20 minutes at 13500 rpm at +4° C, and the supernatant was removed. The pellet had been resuspended to 0.4 ml of TE (pH 8) and 1.5 µl of 20 mg/ml RNase was added and sample were incubated for 5 minutes at +37° C and 1 ml of cold 94% EtOH and 4 µl of 10 M ammonium acetate were added. After mixing sample was centrifuged for 20 minutes at 13500 rpm at +4° C and the supernatant was decanted. Pellet been washed with 0.3 ml of 70% EtOH, dried at room temperature, and resuspended in 50 µl of TE (pH 8). DNA had been stored at +4° C.

4.4 Plasmids and deletion/expression cassettes for *Yarrowia lipolytica*

All PCR reactions were carried out with DyNAzyme II (Finnzymes, Finland), Phusion™ (Finnzymes, Finland) and DyNAzyme Ext (Finnzymes, Finland). All polymerases were used according to manufacturer's protocol. Primers (Sigma-Aldrich, Germany) used in PCR reactions were listed in Table 1. All the restriction enzymes (*KpnI*, *ApaI*, *EcoRI*, *EcoRV*, *HindIII*, *SacI*, *PvuI*, *PmeI*, *XhoI*, *XmnI*) (New England BioLabs, USA) and Fast Digest Enzymes (Fermentas life Sciences, Finland), the T4 DNA ligase (New England BioLabs, USA) and Calf Intestinal Alkaline Phosphatase (Finnzymes, Finland) had been used and all protocols were followed by manufacturer's. Bacterial plasmid purifications were carried out by using QIAprep Spin Miniprep Kit (Qiagen GmbH, Germany). The DNA fragments separation had been carried out by using 1% agarose gels (SeaKem LE agarose BMA, USA). For the extraction of DNA fragments QIAquick Gel Extraction Kit (Qiagen GmbH, Germany) was used. Same kit was also used to purify PCR reactions. Gene Ruler™ 1kb Plus (Fermentas, USA) DNA ladder had been used as a standard marker.

4.4.1 ARS plasmids for *Yarrowia lipolytica*

ARS18 sequence was amplified from *Yarrowia lipolytica* C-00365 genomic DNA with BC1 and BC2 oligo pairs. These oligos were forward and reverse primers with *KpnI* restriction sites. These primers were designed according to available ARS consensus sequences. The amplified sequence had been cloned into pBluescript (B-786) by using *KpnI* restriction enzyme.

Touch down PCR program been used to amplify ARS product: Initialization start of 98°C for 2 minutes, denaturation step at 98°C for 15 sec, annealing step at 65°C for 30 sec

and extension step at 72°C for 1 minute repeated for 13 cycles followed by initialization start of 98°C for 15 sec, annealing step at 55°C for 30 sec and extension step at 72°C for 1 minute repeated for 23 cycles.

Digestion reaction contains 10 µl of 100 ng of insert (PCR product), 1 µl of *KpnI* enzyme, 2 µl of 10 X Fast enzyme buffer, 7 µl of DDIW. The vector digestion has same reagents but insert is replaced by 2 µl of 400 ng of pBluescript (SK-). Restriction mixture were incubated at 37°C for 1 hour.

Reaction was loaded to 1% agarose gel and run at 150 V for 1 hour. UV spectrophotometer was used to visualize the bands and right size insert (1300 bp) and pBluescript vector (2900 bp) were isolated. Bands been transferred into a new 1.5 ml micro centrifuge tube separately and products were extracted by using Gel extraction kit (QIAGEN).

Vector was dephosphorylated by *KpnI* digestion: 1 µg of vector, 2 µl of 10 X Fast Digest Buffer, 1 µl of *KpnI* enzyme, 1 µl of FastAP Thermo sensitive Alkaline Phosphatase, 14 µl of DDIW and incubated at 37°C for 10 minutes. Reaction were arrested by heating at 65°C for 15 minutes and samples been run on agarose gel at 150 V. FastAP Thermo sensitive Alkaline Phosphatase were used to dephosphorylate the vector.

Three different ligation mixtures were prepared with and without insert and vector. Ligation mixture contained 1 µl of vector, 3 µl of insert, 1 µl of T4 DNA ligase buffer, 1 µl of T4 DNA ligase and 4 µl of DDIW. Vector and insert control was same but they were replaced by DDIW. The ligation mixture was incubated at room temperature for 1 hour. 5 µl of ligation mixture had been transferred to 40 µl of chemical competent *E. coli* cells with chemically competent method and plated on ampicillin marker plates. Plasmid pBC7 was constructed.

4.4.2 CRE cassettes

Promoter: TEF1 (elongation factor 1-alpha)

Terminator: Tdh3 (Triose phosphate dehydrogenase)

Cre Recombinase

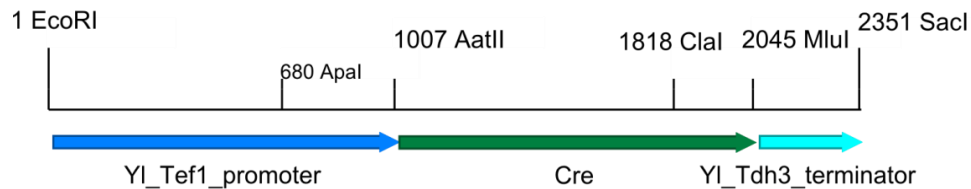


Figure: 7. CRE cassettes

The pBC1 were obtained from Gene Art with Cre recombinase cassette. Cre cassette contains in addition to codon optimised recombinase gene, elongation factor 1-alpha (TEF1) promoter and Triose phosphate dehydrogenase (Tdh3) terminator. This cassette was restricted by using *EcoRI*, *SacI*, *PvuI*. *PvuI* were used to cut pMA plasmid to avoid background. Cre cassette was ligated to B786-pBluescript *EcoRI* and *SacI* sites. These ligations and digestions were performed according to manufacturer's protocol.

Plasmids containing Cre cassettes had been digested with *EcoRI*, *SacI*, *PvuI* : 1 μ l-*EcoRI*, 1 μ l-*SacI*, 1 μ l-*PvuI*, 2 μ l of 10 X Fast enzyme buffer, 5 μ l of 250 ng of insert and 10 μ l of DDIW. The vector digestion contains same reagents except *PvuI* were not added. Insert and vector samples used run on gel at 150 V for 45 minutes. Insert (2356 bp) and vector (2950 bp) were isolated and extracted with Gel extraction kit.

Three ligation reactions had been performed with and without insert and vector: 2 μ l-linear vector DNA, 3 μ l insert DNA, 1 μ l of 10 X T4 ligation buffer, 1 μ l of T4 DNA ligase and 3 μ l of DDW were added. Controls were prepared similarly than above except insert and vector had replaced by DDIW. Ligation mixture was incubated at room temperature for 2 hours. Electroporation had been performed with 2 μ l of ligation mixture which was transferred to 40 μ l of electro competent cells. Electroporation was carried out with Bio-Rad electroporator as described below. After electroporation 100 μ l of mixtures were transformed to LB+ ampicillin plates. pBC5 (pBluescript + Cre) plasmid was constructed.

4.4.3 HPH cassettes

Promoter: Eno₂ (Enolase 2) with LoxP site

Terminator: P_{gk1} (phosphoglycerate kinase) with LoxP site

Hph marker

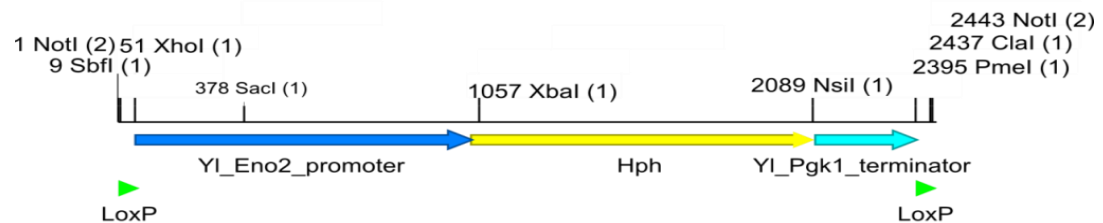


Figure: 8. *HPH cassettes*

The pBC4 had been obtained from Gene Art. *Hph* cassette contains in addition to *Hph* marker gene two LoxP sites at the corners of Enolase 2 (ENO2) promoter and phosphoglycerate kinase (PGK) terminator. This fragment had restricted by using *XhoI*, *PmeI*, *XmnI* were used to cut pMA plasmid to avoid background. *Hph* marker cassette had ligated to pBC5 (Cre) by using *XhoI* and *EcoRV* sites. These had been performed by manufacturer's protocol and transformed into *E. coli* cells.

Hph cassette was cloned into pBluescript + CRE (pBC5). Plasmid containing *Hph* cassettes had been digested with *XhoI*, *PmeI*, *XmnI*: 1 µl-*XhoI*, 1 µl-*PmeI*, 1 µl-*XmnI*. 2 µl of 10 X Fast enzyme buffer, 5.2 µl of 230 ng of insert and 10 µl of DDIW. The pBC5 vector had digested with 1 µl- *XhoI*, 1 µl of *EcoRV* and it contains same reagents. Restriction digestion mixtures were incubated for 1 hour at 37°C. Insert and pBC5 vector samples were run on gel separately at 135 volts for 60 minutes. Insert (2450 bp) and pBC5 vector (5314 bp) was cut and extracted from Gel extraction kit.

Three ligation reactions had been performed with and without insert and vector: 2 µl-linear pBC5 vector DNA, 3 µl insert DNA (*Hph*), 1 µl of 10 X T4 ligation buffer, 1 µl of T4 DNA ligase and 3 µl of DDW was added. Controls were prepared similarly than above except insert and vector had replaced by DDIW. Ligation mixture was incubated at room temperature for 2 hours. Electroporation were performed with 2 µl of ligation mixture which was transferred to 40 µl of electro competent cells. Electroporation had been carried out with Bio-Rad electroporator as describe below. After electroporation 100 µl of mixture was transformed to LB+ ampicillin plates. pBC20 (pBluescript + Cre + *Hph*) plasmid had been constructed.

4.4.4 CRE + Hph + ARS

ARS(pBC7) were cloned into pBluescript+CRE + Hph (pBC20). Plasmid containing pBluescript+Hph cassettes+Cre cassettes and ARS (pBC7) were digested with 1 µl- *KpnI*, 2 µl of 10 X Fast enzyme buffer, 3 µl of 150 ng of insert and 14 µl of DDIW. The pBC20 vector had digested with 1 µl- *KpnI* and it contains same reagents. Restriction digestion mixtures had been incubated for 1 hour at 37°C. Insert(pBC7) and pBC20 vector samples were been run on gel seperately at 135 volts for 90 minutes. Insert (1300 bp) and pBC20 vector (7571 bp) had cut and extracted from Gel extraction kit.

Three ligation reactions were been performed with and without insert and vector: 2 µl-linear pBC20vector DNA, 3 µl insert DNA (pBC7), 1 µl of 10 X T4 ligation buffer, 1 µl of T4 DNA ligase and 3 µl of DDW were added. Controls were been prepared similarly than above except insert and vector were replaced by DDIW. Ligation mixture were incubated at room temperature for 1.5 hours. Electroporation had been performed with 2 µl of ligation mixture which was transfered to 40 µl of electro competent cells. Electroporation was carried out with Bio-Rad electroporator as describe below. After electroporation 100µl of mixtures were transformed to LB+ ampicillin plates. pBC27 (pBluescript + Cre + Hph + ARS) plasmid had been constructed.

4.5 *Nicotiana Tabacum* P-450 hydroxylase and *Arabidopsis thaliana* P450 reductase

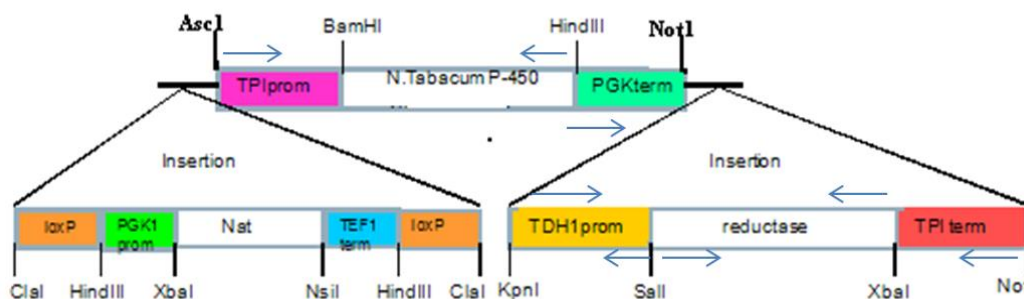


Figure:9. Cloning of overexpression cassette with Nat resistance to *Yarrowia lipolytica*.

Genes to overexpress

Nicotiana tabacum cytochrome P450-dependent fatty acid hydroxylase (CYP94A5) was 1536bps long and it's accession number is AF092916.

Arabidopsis thaliana NADPH-cytochrome P450 reductase was 2079bp long and it had been expressed previously by Tijet et.al, 1998 and Urban et.al, 1997. NCBI Accession number is X66016 The existing over expression cassette were constructed by Jessica Marcon in VTT 2011

Table 1. Primers (Sigma-Aldrich, Germany) used in *Yarrowia lipolytica*.

Name	Sequence	Use
ARS_KpnI_frw	ACTGGGTACCGGATC CCAATATTACACC	amplification of <i>Y.lipolytica</i> ARS with KpnI sites
ARS_KpnI_rev	ACTGGGTACCGATCC AGTCTACACTGAT	amplification of <i>Y.lipolytica</i> ARS with KpnI sites
Tef1_promoter_frw1	AGCCAGACCGATAGC	Colony PCR and for sequencing in order to confirm Cre cassettes
Tef1_promoter_frw2	ATCCGGGTAACCCAT GC	Colony PCR and for sequencing in order to confirm Cre cassettes
Tef1_promoter_rev1	TCGCTCCACTCTACG	Colony PCR and for sequencing in order to confirm Cre cassettes
Cre_frw1	AGGACGTGCGAGACT AC	Colony PCR and for sequencing in order to confirm Cre cassettes
Cre_frw2	GGCCGAACCTAAGACC CTTGTG	Colony PCR and for sequencing in order to confirm Cre cassettes
Cre_frw3	TCTGAGACTGGCGCT ATGGTG	Colony PCR and for sequencing in order to confirm Cre cassettes
Eno2_promoter_frw1	GCGGTCATATCACGCT ACAC	Colony PCR and for sequencing in order to confirm Hph cassettes.
Eno2_promoter_frw2	GGCCATGCAGTCGGA TTTG	Colony PCR and for sequencing in order to confirm Hph cassettes.
Eno2_promoter_rev1	ACTGCCTACACGTTAC C	Colony PCR and for sequencing in order to confirm Hph cassettes.
Hph_frw1	CGTGCTCCGAGTGAA CTCTTG	Colony PCR and for sequencing in order to confirm Hph cassettes.
Hph_frw2	CTCGACGAGCTTATGC	Colony PCR and for sequencing in order to confirm Hph cassettes.
Hph_frw3	GCAACTTCGACGACG	Colony PCR and for sequencing in order to confirm Hph cassettes.
MFE2 3'	TCGTAGGTCATGCCGT TTCCC	Colony PCR and to confirm the deletion of Nat marker.
MFE2 5'	GACCCCTCTACTGATCT CACACTTCC	Colony PCR and to confirm the deletion of Nat marker.
Reductase A.T. 5'	AAGCCCTCAGCGGTT	Colony PCR and to confirm <i>Nicotiana tabacum</i> reductase gene sequence.
Reductase A.T. 3'	GTGGACCAGGGCGT	Colony PCR and to confirm <i>Nicotiana tabacum</i> reductase gene sequence.
TPI term 3'	CAACCGTAGAGGTCC ATGT	Colony PCR and to confirm <i>Nicotiana tabacum</i> reductase gene sequence.
TPI term 5'	TTGGATTAGATGTAC- CGGC	Colony PCR and to confirm <i>Nicotiana tabacum</i> reductase gene sequence.
NTgene5	GAAGATGCCGTTAGA- GAGG	Colony PCR and to confirm <i>Nicotiana tabacum</i> reductase gene sequence.
TDH promo	AAACGCTGCTG- GAGCT	Colony PCR and to confirm <i>Nicotiana tabacum</i> reductase gene sequence.
pTPI 3'	GGGTGGGGTTA- GAAATACAGT	Colony PCR and to confirm <i>Nicotiana tabacum</i> reductase gene sequence.
tPGK 5'	GGATGTGCAGA- TACTTGTACC	Colony PCR and to confirm <i>Nicotiana tabacum</i> reductase gene sequence.
TDH promo	TCGTAGGTCATGCCGT TTCCC	Colony PCR and in order to confirm the deletion of Nat marker.

Jessica Marcon constructed the overexpression cassette and the cassette contains *Nicotiana tabacum* P-450 + *Arabidopsis thaliana* reductase. The cassette was isolated with Plasmid isolation kit were digested with 1 µl *AscI*, 1 µl : *NotI*, 2 µl of 10 X Fast enzyme buffer, 5 µl of 100 ng of insert and 11 µl of DDIW. Restriction digestion mixtures

were incubated for 1.5 hour at 37°C. Incubated samples had been run on gel at 150 volts for 35 minutes. Over expression cassette was cut and extracted from Gel extraction kit.

PLASMID CONSTRUCTION

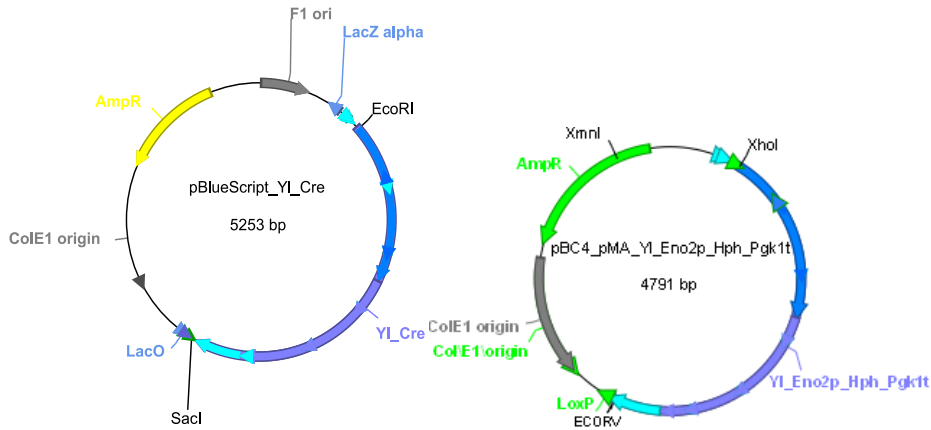


Figure: 10. Plasmid pBC5 (pBluescript+CRE) **Figure: 11.** Plasmid pBC4 (pMA+Hph)

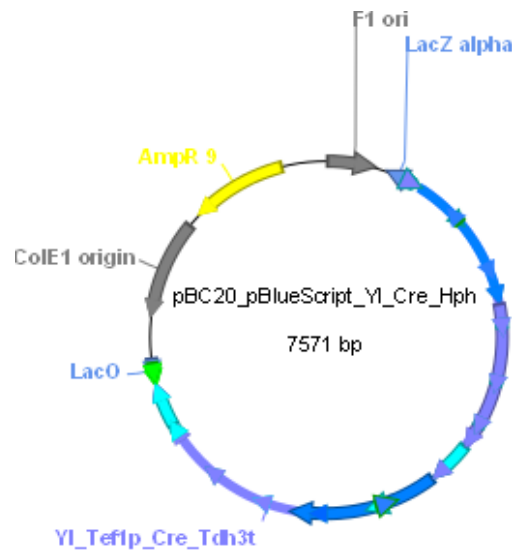


Figure:12. Plasmid pBC20 (pBluescript+Cre+Hph)

The above figure explains the construction of expression plasmid. pBC4 had been restricted with *EcoRV/XhoI/ (XmnI* to cut pMA plasmid) and ligated to pBC5 (As described above). pBluescript with Cre recombinase and Hph marker had been obtained.

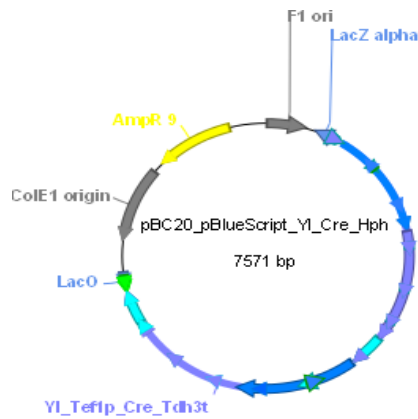


Figure:13. Plasmid pBC20
(pBluecript+Cre+Hph)

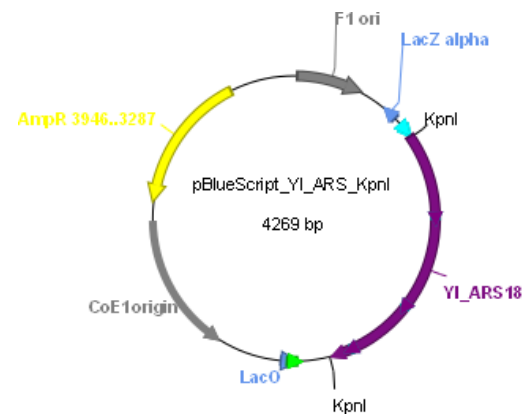


Figure:14. Plasmid pBC7
(pBluescript+ARS)

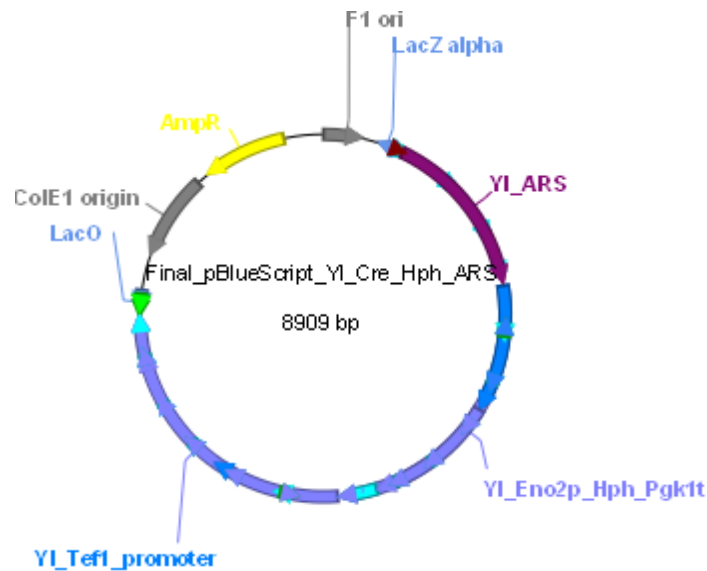


Figure 15. Plasmid pBC27 (pBluescript+Cre+Hph+ARS)

The expression plasmids pBC7 was restricted with *KpnI* and cloned to pBC20 (pBlue-cript+Cre+Hph) as explained above. Final construct (pBluescript+Cre+Hph+ARS) had been obtained.

4.6 Transformations

4.6.1 *E. Coli* transformation

Both chemically competent cells and electro competent cells had been used with bacterial transformations. Chemically competent cells were thawed on ice for 20 minutes and 10 μ l of ligation mixtures were added, chilled on ice for 30 minutes and heat shock was given at +42°C for 30 seconds. 100 μ l of mixtures were plated on LB+ ampicillin resistance plates.

GenePulser apparatus (Bio-Rad, USA) performed bacterial transformations by electrophoresis. 40 μ l of electro competent cells had been used in each transformation. 2 μ l of plasmid DNA was added to electro competent cells. Electroporation were performed by following settings: 25 μ F, 200 Ω and 2.5 kV. After the electroporation, in 0.2 mm electroporation cuvette (BioRad,USA), 1 ml of SOC medium was been added and cells were incubated for one hour in +37° C and shaken it by 250 rpm. LB+ ampicillin plates had been used for plasmid selection.

4.7 STET

Bacterial cell mass were suspended in 50 μ l of cold STET buffer and after 15 minutes vortex, 4 μ l (10 mg/ml) of lysozyme solution had been added. Tubes were incubated for 1 minute at 95 °C heat block and after 10 minutes at 13500rpm centrifugation, pellet been removed by sterilized toothpick and 40 μ l of isopropanol was added to the supernatant, centrifuged for 15 minutes at 13500 rpm. Supernatant had been discarded and pellet has been washed with 200 μ l of 70% ethanol. After centrifugation for 30 seconds at 13500 rpm supernatant were discarded. Pellet were dried completely at room temperature and resuspended in 15 μ l of pH 7.5 of TE buffer.

4.8 *Yarrowia lipolytica* transformation

MFE2 deleted strain 05 were inoculated in 5ml of liquid YPD medium at pH 4 in 100 ml flask. Cells had been grown for 7 hours at +28 °C. After 7 hours cells were been diluted into three different dilutions: 0.1, 1 and 5 μ l in 15 ml of fresh YPD medium at pH 4 in 250 ml flask. These three dilutions were grown overnight at +28 °C. 9×10^7 cells from the culture having OD₆₀₀ = 1- 2 were harvested. After 4 minutes centrifugation at 4000 rpm, cells were washed with 15 ml of sterile water. Supernatant had been discarded and the pellet was resuspended in 0.1 M LiAc at pH 6.0 to cell concentration of 5×10^7 cells/ml. Cells been incubated at 1 hour in gentle shaking at +28°C. Herring sperm were used as a carrier DNA and carrier DNA had been incubated at +100°C for 15 minutes and placed on ice immediately. 100 μ l of cells had been transformed into 1.5 ml eppendorf and after 30 seconds centrifugation, supernatant had discarded. To harvested cells, a mixture of 240 μ l 50% PEG 4000, 36 μ l of 1M lithium acetate at pH 6.0, 5 μ l of 50 μ g of herring sperm DNA, 79 μ l of sterile water and 2 μ g of Cre plasmid DNA (pBC27) i.e., (p Bluescript + Cre + Hph+ ARS) were added. The mixtures were incubated in agitation for 30 minutes at +28°C at 250rpm and heat shock was given at 39°C for 5 minutes. After 60 seconds centrifugation the cells been resuspended in 1 ml of YPD medium and incubated for 3 hours at +28°C. Cells been plated on YPD+Hygromycin (400 μ g/ml) plates.

The overexpression cassette was transformed into *Yarrowia lipolytica* Cre plasmid looped out strain- 1 (*ΔMFE clone 1*). It had been transformed with the same method as above mentioned but the plasmid DNA had replaced by 2 μ g of Over expression cassette

(*Nt P450+At Reductase*) and cells were plated on YPD + Nourseothricin (400 µg/ml) plates.

4.9 Yeast colony PCR

Yeast colony PCR been carried out with BC88 and BC90 oligo pair. This oligo pair is from 5' and 3' non coding regions of *MFE2* gene. PCR reaction mix contained 2 µl of 2 mM dNTP-mix, 2 µl of 10 x Dynazyme Buffer, 13.5 µl of DDW, 2 µl of both 20pmol/ µl concentration of primers and 0.5 µl of Dynazyme II enzyme. Small amount of yeast colony been added in 50 µl of 1 mg ml⁻¹ Zymolase solution and incubated for 10 minutes at 37°C. 2.5 µl of PCR mixture was added to Zymolase treated cells and PCR with 94°C 7 minutes hot start, denaturation 94°C 45 sec, annealing 59°C 30 sec, elongation 72°C 1 minute and repeated for the 34 cycles, final elongation at 72°C 10 minutes were carried out.

To confirm the over expression cassette (*Nt P450+At Reductase*) yeast colony PCR was performed with different pairs of oligos same as above (oligos mentioned in Materials and Methods in Table 3). Reductase 5' and Reductase 3' oligo pair from *Arabidopsis thaliana* P450 reductase. T PGK5' and p TDH 3' oligo pair where TPGK 5' oligo was from terminator of *Nicotiana tabacum* and TDH 3' was from promoter of *Arabidopsis thaliana* P450 reductase. TDH5' and TPI3' oligo pair was from promoter and terminator of *Arabidopsis thaliana* reductase.

4.10 Glycerol stock

Selected yeasts strains were streaked on selective plates and were grown for 3 days at +30°C. A mix of 1 ml of 15% glycerol and 0.9% NaCl solution were taken in Cryo tube and half of yeast growth had been looped and suspended in above buffer. Yeast strains were been stored at -80°C.

Selected *E. coli* strains been grown in 2ml of LB-ampicillin medium at +37° C 250 rpm. 500µl of fresh LB-ampicillin medium had been added to preculture and incubated for 30 minutes at +37° C in 250 rpm. 860 µl of 87 % glycerol, 47 µl of 5 M NaCl, 595 µl of bacterial culture had mixed and incubated at room temperature for 2 hours. Cells were been stored at -80°C.

4.11 Sequencing

DNA sequencing reactions were performed by using Big Dye^R sequencing kit (Applied-Biosystems, USA) as described by Platt et al. (Platt et al., 2007). These reactions were analysed with ABI Prism^R 3100 Genetic analyser (PE/Applied Biosystems, Perkin Elmer, USA).

4.12 Cultivations

The complex SMIT YPD medium used in *Yarrowia lipolytica* cultivation had 1 % Bactopeptone (BD, USA), 1% Bacto-yeast extract (BD, USA) and 2 % of Glucose (Sigma-Aldrich, Germany) at pH 5.5.

Cultivations for diacid productions had been carried out as follows:

Preculture-1 were prepared by adding yeast colony to 4ml of Smit-YPD medium in 15 ml tubes and grown at 250rpm overnight at +28°C or +30°C. Next day 100µl of preculture-1 were added into 10ml of fresh Smit-YPD medium and grown overnight at 250rpm. Following day Growth phase were started by adding 2ml of preculture 2 in 100ml of Smit YPD medium in 500 ml flasks and grown overnight at 250rpm. Following day (Production phase): 10 ml of 30 g/l nonanoate (pelargonic acid) or 1ml of oleic acid was added. 500µl of sample was collected every day from each cultivation and centrifuged at 6000 rpm for 1 minute and supernatant had been collected. Residual glucose amount been checked with Glucose sticks and if glucose were utilized then 0,88g glucose per 1g biomass was been added. In addition, pH was been checked every day and adjusted to pH 8.0 with 4N NaOH. These cultivation steps were followed for 14 days.

Biomass had been calculated by formula [biomass g = $0,1 \times 0,7435 \times \text{OD} \times \text{Volume of 40\% glucose to be added} = \text{biomass} \times 0,88/0.4$]

4.13 High-performance liquid chromatography (HPLC)

High performance liquid chromatography (HPLC) were used to analyse the residual glucose amounts. Supernatant samples collected from cultivations (100 µl) were been diluted in 900 µl (50 mM H₂SO₄). Anninex HPX-87 H organic acid column (300 mm*7.8 mm) and fast acid analysis column (100 mm*7.8 mm) had been used. Columns were maintained at +55°C and 5 mM H₂SO₄ (Merck KGaA, Germany) were used an eluent with flow rate of 0.5 ml min⁻¹. The running time of each sample was 40 minutes. Waters 2695 system with Waters 3410 RI detector (Waters, USA) was used.

4.14 Gas chromatography-Mass Spectrometry (GC-MS)

Supernatant samples (100µl) had been taken into 2ml eppendorf tubes. 50 µl of 1 M HCL was been added and mixed thoroughly. Then 500 µl of TBME and 25 µl of internal standard (TriHeptadecanoic acid (C17:0 about 1µg/µl)) had been added. After 10 minutes vortexing, the samples were placed on ice for 15 minutes. After centrifugation at 13500 rpm for 5 minutes, the upper phase was been transferred into new GC-MS glass tubes. TBME extraction step was repeated by addition of 500 µl of TBME. The upper phase were again collected.

The samples were been trimethylsilylated with MSTFA (*N*-methyl-*N*-trimethylsilyltrifluoroacetamide; Pierce Chemicals, Rockford, IL, USA) at 80 °C for 20 minutes. An

Agilent 7890A GC combined with a 5975C mass selective detector had equipped with an Rtx®-5MS silica capillary column (15m, 0.25 mm ID, 0.25 µm (Restek, Bellefonte, PA, USA) were used to analyses. The oven temperature was increased from 70°C for 1min at a rate of 10°C/min to 270°C for 4min. The split ratio was 20:1 and the samples were injected by a Gerstel Maestro MPS 2 sampling system (Gerstel GmbH&Co.KG, Müllheim an der Ruhr, Germany). The data was collected at a mass range of 40-600 amu. Identification of compounds were based on retention times and mass spectral library comparison (NIST '08, Scientific Instrument Services, Inc., Ringoes, NJ, USA).

4.15 Plasmids and deletion/expression cassettes for *Pichia guilliermondii*

4.15.1 ARS plasmids for *Pichia guilliermondii*

ARS sequence been amplified from *Pichia guilliermondii* C-72064 genomic DNA with BC3 and BC4 primers. These oligos were forward and reverse primers with ApaI sites. The primers were been designed from available published ARS sequences. The amplified sequence were cloned into pBlue script (B-786) by using ApaI restriction enzyme. ARS was constructed by using same Touch down PCR program and same method for cloning as deccribed below. The restriction digestion enzyme is replaced by ApaI.

4.15.2 CRE cassettes for *Pichia guilliermondii*

Promoter: TEF1 (elongation factor 1-alpha)

Terminator: Tdh3 (Triose phosphate dehydrogenase)

Cre Recombinase

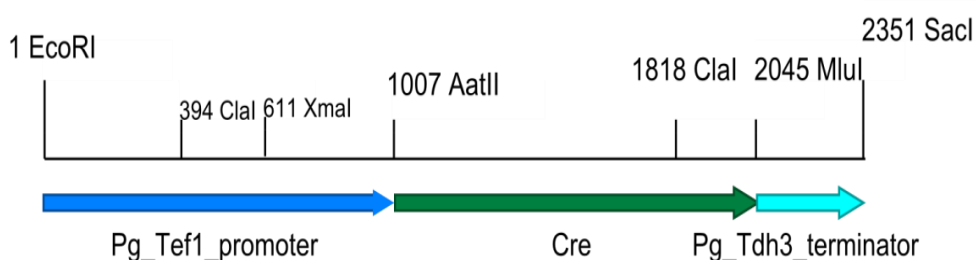


Figure: 16. CRE CASSETTES

The pBC2 was obtained from Gene Art with Cre recombinase cassette. Cre cassette contains in addition to codon optimised recombinase gene, elongation factor 1-alpha (TEF1) promoter and Triose phosphate dehydrogenase (Tdh3) terminator. This cassette were restricted by using *EcoRI*, *SacI*, *PvuI*. *PvuI* was used to cut pMA plasmid to avoid back-

ground. Cre cassette were ligated to B786-pBluescript EcoRI and SacI sites. These ligations and digestions were performed according to manufacturer's protocol. Electroporation was been carried out with Bio-Rad electroporator as describe above. Plasmid pBC6 (pBluescript + Cre) was constructed.

4.15.3 HPH cassettes for *Pichia guilliermondii*

Promoter: Eno2 (Enolase 2) with LoxP site

Terminator: Pgk1 (phosphoglycerate kinase) with LoxP site

Hph marker

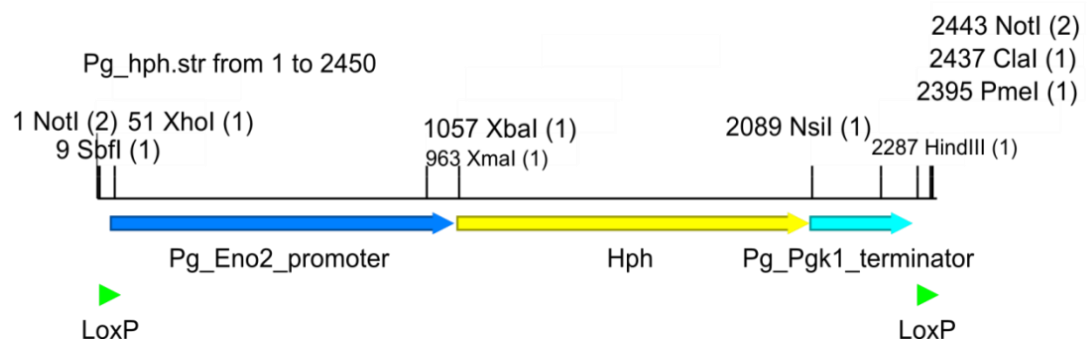


Figure: 17. HPH CASSETTES

The pBC3 was obtained from Gene Art. Hph cassette contains in addition to Hph marker gene two LoxP sites at the corners of Enolase 2 (ENO2) promoter and phosphoglycerate kinase (PGK) terminator. This fragment were restricted by using *XhoI*, *PmeI* and *XmnI*. *XmnI* were been used to cut pMA plasmid to avoid background. Hph marker cassette had been ligated to pBC6 (Cre) by using *XhoI* and *EcoRV* sites. These were performed by manufacturer's protocol and transformed into E.coli cells. Transformation into E. coli had been carried out similarly than in above. Plasmid pBC8 with ARS sequence and Cre cassette were constructed (pBC50).

All the methods like PCR for ARS(3.4.1), Restriction digestion, Agarose Gel electrophoresis, Ligation, Plasmid isolation, Colony PCR, Cre cassettes(3.4.2) were performed same as *Yarrowia lipoytica* and in order to confirm the *Pichia guilliermondii* plasmids Table 3 oligo's were used.

4.16 Gibson assembly

This method had developed by Dr.Daniel Gibson at J.Craig Venter Institute and this method was licensed to New England's BioLabs Inc., NEBuilder (<http://neb-uilder.neb.com/>) were used to design primers for assembling DNA fragments. The primers with overlapping (15-25) nucleotide sequences between two adjacent DNA fragments were designed.

Forward and reverse primers mentioned in Table 2. (primers with overlap for each adjacent DNA fragments) were placed in PCR reaction and PCR product had been amplified by Gibson assembly PCR primers. The 4 DNA fragments (pBluescript, ARS sequence, Cre cassette, Hph cassette) were been prepared for assembly and approximately total 1pmols concentrations were taken according to protocol. This Assembly reaction had been incubated in thermo cycler at 50 °C for 60 minutes. The reaction were transferred into Electro competent cells of DH5 alpha and Top10 by using GenPulser as described above.

Fragment Arrangement

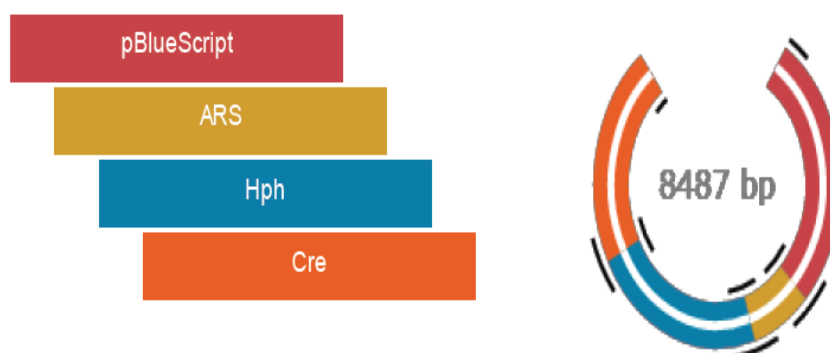


Figure 18 (a)

Figure 18 (b)

Figure18. a.) Explains NEB fragments arrangement for *Pichia guilliermondii* construct and b.)Gibson assembly picture construct for *Pichia guilliermondii*.

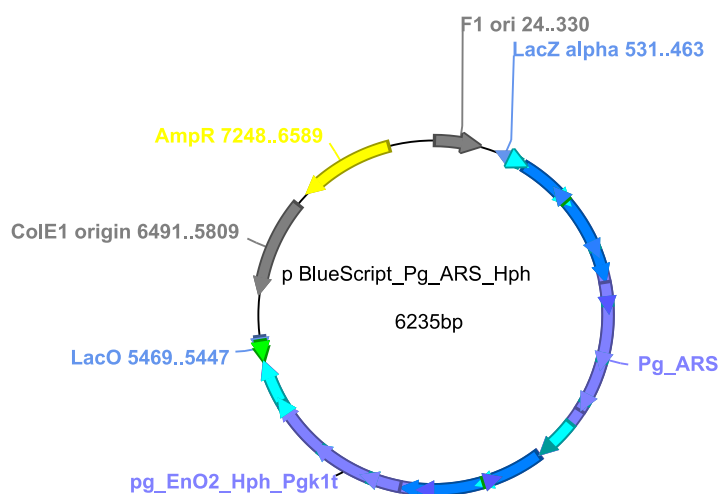


Figure:19. Plasmid pBC50 (pBluescript+ARS+Hph)

Table: 2. List of Primers used in Gibson assembly for *Pichia guilliermondii* constructs.

S.No	Use	Amplify Region
BC190	GGATTGTTGGTCAACGGGCTGCAGGAATTCGAT	pBluescript and Pg ARS flank
BC191	CGACGGTATCGATAAGCTTGATATC GAATTCCTGCAGCCCGTTGACCAACAATCCCGA	Pg ARS and pBluescript flank
BC192	CGAAGTTATCCTGCAGGGCGGCCGC GTCGACACACATCACACG	Pg ARS and Pg Hph flank
BC193	GAGTCACAAGAGCGTGTGATGTGTGTCGAC GCGGCCGCCCTGCAGGAT	Pg Hph and Pg ARS flank
BC194	AGTTTGGTGAATTTTCGGAATT CGCGGCCGCATCGATATAACTTCG	Pg Hph and Pg Cre flank
BC195	CATTATACGAAGTTATATCGATG CGGCCGCGAATTCCGAAAAATTCACC	Pg Cre and Pg Hph flank
BC196	GCGGTGGCGGCCGCTCTAGA ACTAGTGGATCCCCCGAGCTCGCTGAATTTGTG	Pg Cre and pBluescript flank
BC197	TTCACAAATTCAGCGAGCTC GGGGGATCCACTAGTTCTAG	pBluescript and Pg Cre flank
BC198	TATCGATAAGCTTGATATCGAATTCC TGCAGCCCGCGGCCGCCCTGCAGGAT	PgHph, pBluescript and ARS flank
BC199	TATACGAAGTTATCCTGCAGGGCGGCCGC GGGCTGCAGGAATTCGAT	pBluescript and Pg Hph flank

Table: 3. List of primers for constructing *Pichia guilliermondii* plasmids

Name	Sequence	Use
Pg_ARS_ApaI_frw	ACTGGGGCCCGTTGAC- CAACAA TCCCGA	amplifying <i>P.guilliermondii</i> ARS with ApaI sites
Pg_ARS_ApaI_rev	ACTGGGGCCCGTCGACACAC ATCACACG	amplifying <i>P.guilliermondii</i> ARS with ApaI sites
Pg_Tef1_promoter_frw1	CGCGACGAACAAGATGATG	Colony PCR and to confirm Cre cassettes
Pg_Tef1_promoter_frw2	GAGAAGGAAGCCGCCGAATTG	Colony PCR and to confirm Cre cassettes
Pg_Tef1_promoter_rev1	CGAGGCGACGGGAACCTTTATG	Colony PCR and to confirm Cre cassettes
Pg_Cre_frw1	CGAGCCAGAGGATGTGAGAG	Colony PCR and to confirm Cre cassettes
Pg_Cre_frw2	CATCGGAAGAACTAAGACC	Colony PCR and to confirm Cre cassettes
Pg_Cre_frw3	CATGGTGCGGTTGTTG	Colony PCR and to confirm Cre cassettes
Pg_Eno2_promoter_frw1	GAGTGGAGCGATTATGC	Colony PCR and to confirm Hph cassettes
Pg_Eno2_promoter_frw2	CAGAGCCATTGATTACATCG	Colony PCR and to confirm Hph cassettes
Pg_Eno2_promoter_rev1	GATTCGCAGCCATAGTATTG	Colony PCR and to confirm Hph cassettes
Pg_Hph_frw1	TTAACTCCTGTGCTGACG	Colony PCR and to confirm Hph cassettes
Pg_Hph_frw2	GAATTGATGTTGTGGGCTG	Colony PCR and to confirm Hph cassettes
Pg_Hph_frw3	CCGCTGCTGTTTGGACTGAC	Colony PCR and to confirm Hph cassettes

5. RESULTS

5.1 *Yarrowia lipolytica*

Table 4. The below table shows plasmids constructed in this work:

Name	Plasmid name	Backbone	Cloning sites	selection	strain
pBC7	pBlue_Yl_ARS	pBluescript	KpnI	Ampicillin	DH5 α
pBC5	pBlue_Yl_Cre	pBluescript	EcoRI/SacI	Ampicillin	DH5 α
pBC20	pBlue_Yl_Cre_Hph	pBluescript	EcoRV,PmeI/XhoI	Ampicillin	DH5 α
pBC27	pBlue_Yl_Cre_Hph_ARS	pBluescript	KpnI	Ampicillin	DH5 α

5.1.1 ARS-plasmid (pBC7)

An ARS18 sequences were been amplified by using BC1 and BC2 oligo pair from *Yarrowia lipolytica*C-00365 strain. These primers were designed based on available published sequence of ARS18. Clear amplified bands were been obtained when compared to negative control and the band size is 1300base pairs as expected. These were compared with 1kb Gene Ruler DNA ladder. Thus, the verified PCR products were been successful and confirmed by sequencing after cloned into pBluescript plasmid.



Figure 20. The above gel picture explains the ARS18 sequence band and Ladder was on both the sides of a gel. Lane1-Lane9 contains amplified products of *Yarrowia lipolytica* and expected band is 1300bp. In the last row NC refers to negative control in PCR reaction with water.

5.1.2 CRE-plasmid (pBC5)

Synthesised CRE cassette had been cloned into pBluescript (SK-). The Colony PCR check were performed to the selected colonies with M13 forward and BC55 primers. M13 forward primer is from pBluescript plasmid and BC55 is a reverse primer of Tef1 promoter from Cre cassette. Clear bands were obtained with 400bp as expected when compared with 1kb Gene Ruler.



Figure 21. A colony PCR performed gel picture were seen and two kb-ladders were been placed on both the corners of the gel. Lane 1-Lane 24 has CRE colonies but Lane-15 had negative control. A clear band of 400bp was obtained.

5.1.3 HPH + CRE - plasmid (pBC20)

Synthesised Hph cassette were cloned into pBluescript+CRE plasmid(pBC5). Colonies were been verified by performing restriction digestion with *EcoRI* and *XhoI*. The expected band sizes were 5220bp and 2351 bp. Lane 3, Lane 12 and Lane 24 have two bands with expected band sizes. The verification been successful and plasmids were confirmed by sequencing.



Figure 22. The upper gel and lower gel kb- ladder on last rows of gel. Lane 1-Lane 28 were colonies (pBluescript+Cre+Hph) performed Restriction digestion. The expected bands were 5220bp and 2351bp

5.1.4 ARS+CRE+Hph -plasmid (pBC27)

ARS18 cassette from pBC7 plasmid had been cloned into pBC20. Colonies were screened by performing restriction digestion with *EcoRI* and *HindIII*. A band size of 5800bp and 3100bp were expected and they were seen in Lanes 4,6,9,12,24,25,26 and 28. The verification was successful and observed expected bands clearly. The constructed plasmids were confirmed by sequencing.

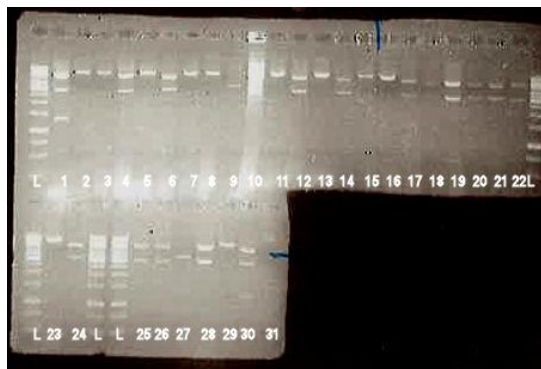


Figure 23. The gel had GeneRuler 1Kb kb-ladder at all the last rows of gel. Lanes 1 – Lanes 30 were colonies (pBluescript ARS+CRE+Hph) performed Restriction Digestion and bands expected were 5800bp and 3100bp

5.2 Nourseothricin marker and CRE plasmid loop Out

Cre recombinase constructed plasmids (pBC27) tested by transforming it to *ΔMFE2 Yarrowia lipolytica* strain. Colonies were observed on YPD+Hygromycin (400μg/ml) plates. Selected transformants having pBC27 plasmid were cultured in 10ml YPD+Hygromycin (400μg/ml) for three days. Fresh culture was prepared every day from overnight culture. Serial dilutions with 0.9% NaCl had been plated on YPD+Triton-X plates. Colonies from YPD+Triton-X plates were streaked on YPD, YPD+hygromycin (400μg/ml) and YPD+Nourseothricin (400μg/ml) plates.

Totally 20 colonies been verified. All colonies were grown on YPD medium, but colonies 1,6,12,13,18,19 did not grow on YPD+Hygromycin (400μg/ml) and no colonies had grown on YPD+Nourseothricin (400μg/ml) plates. This indicates there had been a successful loop out of nourseothricin (400μg/ml) marker from genomic DNA and also Cre recombinase plasmid was looped out from some of the *MFE2* deleted *Yarrowia lipolytica* transformants.

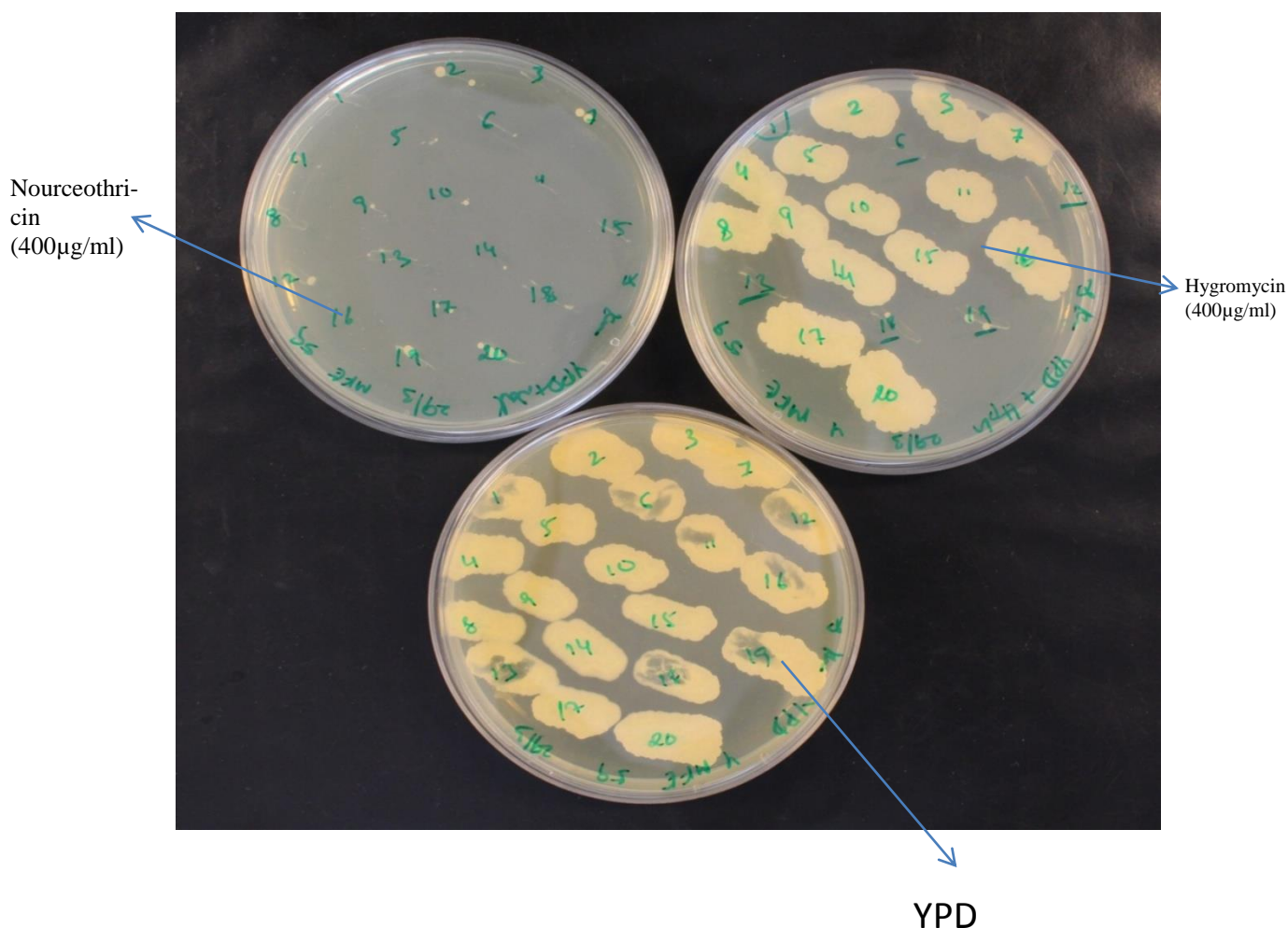


Figure 24 . Left agar plate had YPD+Nourseothricin (400µg/ml) and right plate had YPD+Hygromycin (400µg/ml) and down plate contains only YPD mediu

5.2.1 PCR confirmation of the Nat marker loop out transformant

Yeast colony PCR were been performed with the selected loop out transformants with BC88 and BC90 oligos. BC88 had a nucleotide sequence from MFE 5' flank forward and BC90 has MFE 3' flank reverse. These oligo's were from outside to MFE2 deletion cassette in order to confirm whether nourseothricin marker had been looped out. .

A 832bp fragment were expected and they had obtained in all colonies. But Lane 1, Lane 4 and Lane 5 had bright bands whereas Lane 2, Lane 3 and Lane 6 had pale bands. Lane-7 is a negative control without any band. This indicates there was been a successful loop out of nourseothricin marker from the above selected colonies.



Figure 25. The figure explains L-ladder on the both corner sides of the gel. Lanes 1-6 shows clear bands of 832bp and Lane-7 had a NC-negative control.

5.2.2 PCR confirmation of the *Nicotiana Tabacum* P-450 hydroxylase and *Arabidopsis thaliana* P450 reductase

To over express the cytochrome P450 complex in *MFE2* deleted strain, cassette containing *Nicotiana tabacum* P-450 hydroxylase and *Arabidopsis thaliana* reductase were transformed in *Y. lipolytica*. Primers NT gene 5' and pTPI 3' had been used to check colony PCR. NT gene 5' oligo is from *Nicotiana tabacum* gene and pTPI 3' represents promoter TPI of *Nicotiana tabacum*. This oligo pair is chosen from inside the cassette because it's hard to find where the cassette was attached in the whole genome. There are other oligo pairs to check the complete cassette.



Figure 26. The very left and right corner L-indicates ladder. Lane 1 to Lane 14 were different colonies. Lane-15 was been a positive control. When compared to Positive control

Lane-1 shows the equal base pairs. A clear band of 640base pairs was expected and seen.

To reconfirm the above results yeast colony PCR were performed for three colonies with different oligo pairs (oligos mentioned in Materials and Methods in *Table 3*). All the oligo pairs showed the positive results. Reductase 5' and Reductase 3' oligo pair from *Arabidopsis thaliana* P450 reductase. T PGK5' and pTDH 3' oligo pair where TPGK 5' oligo was from terminator of *Nicotiana tabacum* and TDH 3' was from promoter of *Arabidopsis thaliana* P450 reductase. TDH5' and TPI3' oligo pair was from promoter and terminator of *Arabidopsis thaliana* reductase. This indicates the presence of *Nicotiana Tabacum* P-450hydroxylase and *Arabidopsis thaliana* reductase gene.



Figure 27. In the above gel picture L-indicates Ladder and Lanes 1, 2, 3 all were positive with a clear band of 1500base pairs. A combination of Reductase5' and Reductase3' oligo proved the presence of the Reductase gene in MFE deleted loop out marker strains. This indicates the integration of Cytochrome P450 complex cassette was been successful.

5.2.3 *Yarrowia lipolytica* ARS

The *Yarrowia lipolytica* sequences had been analysed by multiple sequence alignment. There were two published sequences available for *Yarrowia lipolytica*. Namely, ARS18 and ARS68. A comparison were performed to both published sequences and with pBC7 sequence from this work with Clustal W (Multiple sequence alignment).

1-*Yarrowia_70* -This represents pBC7 sequence from this work.

2-*Yarrowia_18ARS*-This represents published sequence of ARS18 available from PubMed.

3-*Yarrowia_68ARS*-This represents ARS68 published sequence from PubMed.

Pairwise alignment showed that ARS sequence in pBC7 to 18ARS was more homology with a great score of 99.69. Whereas ARS sequence in pBC7 and *Yarrowia_68ARS* alignment score was 64.56. This indicates that ARS sequence of this work was more related to *Yarrowia lipolytica* ARS18 sequence. This shows pBC7 was almost equal to ARS18.

Table 5. Scores Table of ARS *Yarrowia lipolytica*

SeqA ♦	Name ♦	Length ♦	SeqB ♦	Name ♦	Length ♦	Score ♦
1	1-Yarrowia_70>	1332	2	2-Yarrowia_18ARS>	1305	99.69
1	1-Yarrowia_70>	1332	3	3-Yarrowia_68ARS>	2309	64.56
2	2-Yarrowia_18ARS>	1305	3	3-Yarrowia_68ARS>	2309	64.67

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1-Yarrowia_70>GGATCCCAATATT--ACACCCAA-----GTAGCATG      29
2-Yarrowia_18ARS>GGATCCCAATATT--ACACCCAA-----GTAGCATG      29
3-Yarrowia_68ARS>GGATCCGAATTTCAGAACCTAATTTATCTGTTACCCGGCCTGTGGCTCG      50
          ***** ** *          ** *          ** *

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1-Yarrowia_70>CATAAGCTAAAAGT----AACTCGCAGCG-----CAC      57
2-Yarrowia_18ARS>CATAAGCTAAAAGT----AACTCGCAGCG-----CAC      57
3-Yarrowia_68ARS>CACAGCTTAAAAATGGTCAAACCTTCCCTTCTTGTCTTTTTTTCCTCAC      100
          ** *      ***** *          **** * *          ***

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1-Yarrowia_70>ACCGTGCAGATTC-----ATAAGTCTATGATTA-----      85
2-Yarrowia_18ARS>ACCGTGCAGATTC-----ATAAGTCTATGATTA-----      85
3-Yarrowia_68ARS>ATTCAATCAGGTTCTTGTCTTGATCTTTCAAGTGAGTAT-TAATTACCGAC      149
          *          *** **          * *** * * *****

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1-Yarrowia_70>-----ATTGAACGCC-----AATAACC-----      102
2-Yarrowia_18ARS>-----ATTGAACGCC-----AATAACC-----      102
3-Yarrowia_68ARS>CTTGGTTCTTCATTGGGAGAGCATTGGAAGCCGTGGTGCAGCAACCACAA      199
          **** * ***          *      ****

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1-Yarrowia_70>--CGGCT-----TACTACA-----AGTACAAGTAG      125
2-Yarrowia_18ARS>--CGGCT-----TACTACA-----AGTACAAGTAG      125
3-Yarrowia_68ARS>AACGGTTCTTCCCTTCGATACCTTCTTGCCTGCCTTTCAATACAAGTCG      249
          *** *          * * * *          * ***** *

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1-Yarrowia_70>GTATACATAGCGGT-----      139
2-Yarrowia_18ARS>GTATACATAGCGGT-----      139
3-Yarrowia_68ARS>GCTCGATTAGCGGTGGTCGCCCCGCCAGCGGAGAACATGGAACATAACCC      299
          *          **** *

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1-Yarrowia_70>--AATGAA--TCATTAGAACAAAAAACAACAA-----AAA      173
2-Yarrowia_18ARS>--AATGAA--TCATTAGA--AAAAAACAACAA-----AAA      171
3-Yarrowia_68ARS>AGAATGAGAGCTAAGTGGAGAAAGAAGAGAGTCAGACGACTCAAGCGAAA      349
          ***** * * * * * ** * * * * ** *          ***

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1-Yarrowia_70>-----AAACAA-----AAC-----      182
2-Yarrowia_18ARS>-----AAACAA-----AAC-----      180
3-Yarrowia_68ARS>GCGCCGCAAGGTCCGAGCTCGATCCAATAAGCGGTTTTTAACGGAGATT      399
          *** **          ***

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1-Yarrowia_70>-----AAACTG-----TTGTGGATG---CATC      201
2-Yarrowia_18ARS>-----AAACTG-----TTGTGGATG---CATC      199
3-Yarrowia_68ARS>TAACACTAAATCGAAGAACTTTTCCGTTTCATTTGCGAATGAGCTCGTT      449
          *** *          *** * ***          * *

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1-Yarrowia_70>AACAG-----TAGTAC      212
2-Yarrowia_18ARS>AACAG-----TAGT--      208
3-Yarrowia_68ARS>AACAAATCCCCAGTTTTTTTATCCAGCTGTAAGGATTGACATTAGTAA      499
          ****          ****

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1-Yarrowia_70>ATAGTTGTACAGTAGTACAT-----AGTT----- 236
2-Yarrowia_18ARS>-----ACAT-----AGTT----- 216
3-Yarrowia_68ARS>TGAATTATTGTTTGGTATATTTAAATCTGTAGTTCCTTTCTGTCCGTGTC 549
                      *  **                      ****

1-Yarrowia_70>-----GTAC---GATGTACTTGTACTTGTAAA-----AG 262
2-Yarrowia_18ARS>-----GTAC---GATGTACTTGTACTTGTAAA-----AG 242
3-Yarrowia_68ARS>GGCAACTGTCGTACTCGTGATTTACTTGTATTGACGAATACTTACTGTAG 599
                      ****      *** ***** *      **      **

1-Yarrowia_70>C-----AAAAATGTACAATATC----- 279
2-Yarrowia_18ARS>C-----AAAAATGTACAATATC----- 259
3-Yarrowia_68ARS>CGCACTCTGCTGCTACTGGTCGTAAGGATGTGCTATTTCCGGTGTATGGTG 649
                      *                      ** ***** * ** **

1-Yarrowia_70>-----TCAGGGAGCGCAAC-----TTTTAC----- 299
2-Yarrowia_18ARS>-----TCAGGGAGCGCAAC-----TTTTAC----- 279
3-Yarrowia_68ARS>GGTTTTTTGGGGGTGCGAACCGAAGACTGTTACACGGGCACGGCTCGTTG 699
                      *   ***   **   ***           *   *****

1-Yarrowia_70>----- 299
2-Yarrowia_18ARS>----- 279
3-Yarrowia_68ARS>TGACACGCACAGAGCTCTTGCGAGTCATGTTGTAGCTAGCTCGTCGTGT 749

1-Yarrowia_70>-----GTTCAAGA-----ACAATGTACCGC 320
2-Yarrowia_18ARS>-----GTTCAAGA-----ACAATGTACCGC 300
3-Yarrowia_68ARS>TCAGGAAGTTCGATGGTTCGAGAGAGTCGCCGCCGAGAACATAC-GC 798
                      ***** ***           * **   *** **

1-Yarrowia_70>GTACCG-----CATTCTGG-----A 335
2-Yarrowia_18ARS>ATACCG-----CATTCTAG-----A 315
3-Yarrowia_68ARS>GCACCGATGTCAGCAGACAGCCTTATTACAAGTATATTCAAGCAAGTATA 848
                      ****                      ***** *      *

1-Yarrowia_70>TTC-TGCGGAACGTCTAACCTGGAATACGATT----- 367
2-Yarrowia_18ARS>TTC-TGCGGAACGTCTAACCTGGAATACGATT----- 347
3-Yarrowia_68ARS>TCCGTAGGGTTCGGGTGATTTGGATCTAAGTTTCGTACTCAACACTCACG 898
                      * * *   **   **   * *   ***** ** * **

1-Yarrowia_70>-----TTTTTTTTCTTTTCATTTTTTT----- 388
2-Yarrowia_18ARS>-----TTTTTTTTCTTTTCATTTTTTT----- 368
3-Yarrowia_68ARS>AGCAGCTTGCCTATGTTACATCCTTTTATCAGACATAACATAATTGGAGT 948
                      **      * * ** *** *****

1-Yarrowia_70>-TGCTTCTTCAAAAG-----TATGTAATTTCTTACCATTACAGT- 427
2-Yarrowia_18ARS>-TGCTTCTTCAAAAG-----TATGTAATTTCTTACCATTACAGT- 407
3-Yarrowia_68ARS>TTACTTACACACGGGGTGACCTGTATGAGCACCACCTACAATTGTAGCA 998
                      * ***   **   *           ***** *      ***** **

1-Yarrowia_70>-TGACACT-----GAACGA-----GGGGGGATTGAA 452
2-Yarrowia_18ARS>-TGACACT-----GAACGA-----GGGGGGATTGAA 432
3-Yarrowia_68ARS>CTGGTACTTGTACAAAGAATTTATTCGTACGAATCACAGGGACGGCCGCC 1048
                      **   ***                      * *****   *** *      *

1-Yarrowia_70>TTA-----AGCAAAA-----ATTAAATCAA----- 474
2-Yarrowia_18ARS>TTA-----AGCAAAA-----ATTAAATCAA----- 454
3-Yarrowia_68ARS>CTCACCGAACCAGCGAATACCTCAGCGGTCCCCTGCAGTGAAGTCAACAAA 1098
                      * *           *** ** *                      * * * *****

1-Yarrowia_70>-----AATACCTTT--ATG-TATCCAGCCCATG-----TA---ATA 504

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2-Yarrowia_18ARS>-----AATACCTTT--ATG-TATCCAGCCCATG-----TA----ATA 484
3-Yarrowia_68ARS>GCGATATGAACATCTTGCGATGGTATCCTGCTGATAGTTTTTACTGTACA 1148
                ** * ***      *** ***** ** **      **      * *

1-Yarrowia_70>AACA-----AAAG 512
2-Yarrowia_18ARS>AACA-----AAAG 492
3-Yarrowia_68ARS>AACACCTGTGTAGCTCCTTCTAGCATTTTTTAAGTTATTCACACCTCAAG 1198
****                      ** *

1-Yarrowia_70>G-----ATTATATAA-----CAAGAAA-TAAATA 535
2-Yarrowia_18ARS>G-----ATTATATAA-----CAAGAAA-TAAATA 515
3-Yarrowia_68ARS>GGAGGGATAAATTAAATAAATTCCAAAAGCGAAGATCGAGAACTAAAT- 1247
                *      *****      * ***** *****

1-Yarrowia_70>TATACCTTTAA-----TGGATCATTA-----GAATAAAAAATAATA 570
2-Yarrowia_18ARS>TATACCTTTAA-----TGGATCATTA-----GAATAAAAAATAA-- 548
3-Yarrowia_68ARS>TAAATTCCAAAAACGAAGTTGGAACACAACCCCCCGAAAAAAACAAC- 1296
                ** * * **      ***** ** *      *** ***** **

1-Yarrowia_70>AATACGAGAAGCACACCAGAG-AAGC-----TTTTTGATTG 605
2-Yarrowia_18ARS>-ATACGAGAAGCACACCAGAG-AAGC-----TTTTTGATTG 582
3-Yarrowia_68ARS>-AACAACAAAACCCAAACAAATAAACAAAACAAAATAAATATATAACTA 1345
                * ** * ** * ** * ** *      * * * * *

1-Yarrowia_70>CCACTATACCGCTA---CTTTGGTAT--ATTCTTATTATAATT----- 643
2-Yarrowia_18ARS>CCACTATACCGCTA---CTTTGGTAT--AT-CTTATTATAATT----- 619
3-Yarrowia_68ARS>CCAGTATCTGACTAAAAGTTCAAACTACTCGTACTTACAACAAATAGAAAT 1395
                *** **      ***      *      **      * ***** * ** *

1-Yarrowia_70>-----GTTGAA--TTTGCAAGATAGAATGTGC--CATTCATTGG--AG 680
2-Yarrowia_18ARS>-----GTTGAA--TTTGCAAGATAGAATGT--CATTCATTGG--AG 654
3-Yarrowia_68ARS>GAGCCGGCCAAAATTCTGCAGAAAAAAATTTCAAACAAGTACTGGTATAA 1445
                *      **      * ***** * * *** *      **      * *** *

1-Yarrowia_70>AGAAATCGAAGGAATATGTGGGA-TGAAATGAC--TAGAAGT----- 719
2-Yarrowia_18ARS>AGAAATCCAAGGAATATGTGGGA-TGAAATGAC--TAGAAGT----- 693
3-Yarrowia_68ARS>TTAAATT-AAAAACACATCAAAGTATCATAACGTTAGTTATTTTATTTT 1494
                ****      **      ** * *      * *      ** **      ***      *

1-Yarrowia_70>-----ATGAACAATGAGAATAGTACATACTTGTACCTGT--ATTTCTAGA 762
2-Yarrowia_18ARS>-----ATGAACAATGAGAATAGTACATACTTGTACCTGT--ATTTCTAGA 736
3-Yarrowia_68ARS>ATTTAATAAAAGAAAACAACAAGATGGGCTCAAACCTTTCAACTTATACG 1544
                ** **      *      * ** *      *      **      * ** * * **

1-Yarrowia_70>AGAGA----GAAAGACAGTTGAGTGT-----GTGATTCTCGTC--CA 798
2-Yarrowia_18ARS>AGAGA----GAAAGACAGTTGAGTGT-----GTGATTCTCGTC--CA 772
3-Yarrowia_68ARS>ATACATACCAATAACAATTTAGTATTTATCTAAGTGCTTTTCGTAGATA 1594
                * * *      **      *** ** * ** *      *** ** *****      *

1-Yarrowia_70>AT--AATA-----ATCTCAATAGTA-ACGTGTGAATAGCTGTTCTTTG 838
2-Yarrowia_18ARS>AT--AATA-----ATCTCAATAGTA-ACGTGTGAATAGCTGTTCTTTG 812
3-Yarrowia_68ARS>ATGGAATACAAATGGATATCCAGAGTATACACATGGATAG-TATACACTG 1643
                **      ****      ** ** * ***** **      ** ***** * * * **

1-Yarrowia_70>ATAGTTGATATTTCTCGATGACTATTTATGTTG-----TACAAGGGATT- 882
2-Yarrowia_18ARS>ATAGTTGATATTTCTCGATGACTATTTATGTTG-----TACAAGGGATT- 856
3-Yarrowia_68ARS>ACA--CGACAATTCTGTAT--CTCTTTATGTTAACTACTGTGAGGCATTA 1689
                * *      ** * ***** **      ** *****      *      *** **

1-Yarrowia_70>-----TTTTTCGTT--GCTGT-TGA----TTTCG 904
2-Yarrowia_18ARS>-----TTTTTCGTT--GCTGT-TGA----TTTCG 878

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3-Yarrowia_68ARS>AATAGAGCTTGATATATAAAATGTTACATTTTCACAGTCTGAACCTTTTGCA 1739
                      *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *

1-Yarrowia_70>AATTAGGCAATGC---AGATATCATTTATG--CT-----AT 935
2-Yarrowia_18ARS>AATTAGGCAATGC---AGATATCATTTATG--CT-----AT 909
3-Yarrowia_68ARS>GATTACCTAATTTGGTAAGATATTAATTATGAAGTGAAGTTGATGGCAT 1789
                      *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *

1-Yarrowia_70>CCATA--TTT-----AAGAT---TTC-----CCA 954
2-Yarrowia_18ARS>CCATA--TTT-----AAGAT---TTC-----CCA 928
3-Yarrowia_68ARS>CCCTAAATTTGATGAAAGATGAAATTGTAAATGAGGTGGTAAAAGAGCTA 1839
                      *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *

1-Yarrowia_70>TACGCATTT-----ATAACATTTATTCTACATAAAT--TGTTAAA 992
2-Yarrowia_18ARS>TACGCATTT-----ATAACATTTATTCTACATAAAT--TGTTAAA 966
3-Yarrowia_68ARS>CAGTCGTTTTGTTTTGAGATACCATCATCTCTAACGAAATATCTATTAAA 1889
                      *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *

1-Yarrowia_70>TGAACGAACTGCCATTATAAATTGTTTCCTAAATAGGAAGTGTTTTCAT 1042
2-Yarrowia_18ARS>TGAACGAACTGCCATTATAAATTGTTTCCTAAATAGGAAGTGTTTTCAT 1016
3-Yarrowia_68ARS>-AATCTCAGTGTGATCATGAGTCATTGCCATCCTGGAAAATGTCAT-CAT 1937
                      *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *

1-Yarrowia_70>AAAGCAAGTA-----AGTTGTCTAATAATACTAAGTA-ATA----- 1077
2-Yarrowia_18ARS>AAAGCAAGTA-----AGTTGTCTAATAATACTAAGTA-ATA----- 1051
3-Yarrowia_68ARS>G--GCTGATATTTCTAAGTGTCTTACTTGAGATAAATATATATTTACAAGA 1985
                      *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *

1-Yarrowia_70>-----AAAATAAGTTCA--TACAATATATTT-TGAGAACATCATTT 1115
2-Yarrowia_18ARS>-----AAAATAAGTTCA--TACAATATATTT-TGAGAACATCATTT 1089
3-Yarrowia_68ARS>ACTTCCCTTGAAATTAATTTAGATATAAAATGTTTGCAGGCAAGTTACTA 2035
                      *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *

1-Yarrowia_70>GGAGGCGGTAGATGGAGTCTGTTTATTA---TTA--AACA----- 1150
2-Yarrowia_18ARS>GGAGGCGGTAGATGGAGTCTGTTTATTA---TTA--AACA----- 1124
3-Yarrowia_68ARS>CGAGG-AATAAATTATATCTGTTGACTAGAAAGTTATGAACATTCAGTATA 2084
                      *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *

1-Yarrowia_70>-ATGCGAG---ATGACC--CCTTAAAT-----ATTGAGAACATCAGT 1186
2-Yarrowia_18ARS>-ATGCGAG---ATGACC--CCTTAAAT-----ATTGAGAACATCAGT 1160
3-Yarrowia_68ARS>TATGCACATATAATAACCAACTTCGAATTTTCCAAATTAGGAA-GTAAAT 2133
                      *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *

1-Yarrowia_70>TG--GAGGCGGCAGATGG-----AGTCTGTCTATTTAGCAATGGGACATG 1229
2-Yarrowia_18ARS>TG--GAGGCGGCAGATGG-----AGTCTGTCTATTTAGCAATGGGACATG 1203
3-Yarrowia_68ARS>TGTTATAATGAACATAGTTTTAGGTTTTATATATTATAATGGAATACG 2183
                      *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *

1-Yarrowia_70>ACTGTCACGTATCATCATGATGTA-TATATATAAT-----ACATA 1268
2-Yarrowia_18ARS>ACTGTCA-GTATCATCATNATGTA-TATATATAAT-----ACATA 1241
3-Yarrowia_68ARS>TCT-CCA-TTATTTTCGCCATCTAATTTATTTAATGAAAAAGTTAGATA 2231
                      *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *

1-Yarrowia_70>-----TAATATTATATAACACGATTTTTTTT-----AAATTATTGGC 1304
2-Yarrowia_18ARS>-----TAATATTATATAACACGATTTTTTTT-----AAATTATTGGC 1277
3-Yarrowia_68ARS>GATGATCTAATTTTATATATTAAATAGATGGTGAGAGAAAAAATGGC 2281
                      *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *

1-Yarrowia_70>CCGAAAATTAATCAGTGTAGACTGGATC- 1332
2-Yarrowia_18ARS>CCGAAAATTAATCAGTGTAGACTGGATC- 1305

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3-Yarrowia_68ARS>TTGCATATATATATATATATAC-AGATCC 2309
 * * * * * * * * * *

5.3 Cultivations

The strains used in cultivations were: *Yarrowia lipolytica* wild type C-00365, Δ MFE 1 and Δ MFE12, which were two different Δ MFE2 strains from nourseothricin marker loop out experiment. Δ MFE+ Nt Rd+P450-16(1), Δ MFE+ Nt Rd+P450-16(2), Δ MFE+ NtRd+P450 clone 1 and Δ MFE+ NtRd+P450 clone 4 strains which were Δ MFE2 loop out transformants having *Nicotiana Tabacum* P450 hydroxylase and *Arabidopsis thaliana* P450 reductase complex expressed.

5.3.1 HPLC (High Performance liquid Chromatography)

High-performance liquid chromatography (HPLC) had been used to check the glucose level in the six different strains. Tested strains were been cultured for 120 hours and 336 hours on YPD medium with 0.3 % Pelagronic acid as a substrate. Another cultivations were made with 1 % Oleic acid with the same strains.

HPLC results of pelagronic acid and oleic acid of three day cultivation shows that there were complete utilization of glucose after 47 hours, 91 hours and 164 hours cultivation with modified Δ MFE and Δ MFE+ At red+ Nt P450 strains on three day cultivation. So, based on glucose consumption these different time points were been chosen and performed with Gas Chromatography-Mass spectrometry.

Cultivations were been repeated at two different temperatures 28°C and 30°C fourteen days with both pelagronic acid and oleic acid with all six different strains which were mentioned above. At 28° C on 168 hour the Δ MFE+ At red+ Nt P450 strain has utilized glucose completely and therefore time points 168 hours, 216 hours and 312 hours were been chosen. Whereas at 30° C all glucose were consumed after 72 hours. So timepoints 72 hours, 120 hours, 168 hours, 216 hours, 240 hours and 288 hours had been chosen.

According to HPLC results from Oleic acid cultivations at 28°C and 30°C glucose were completely consumed after 24 hours for Δ MFE4C and Δ MFE+ At red+ Nt P450. So time points 24 hours, 72 hours, 120 hours and 192 hours at 28°C had been chosen. At 30°C glucose had been utilized completely at 24 hours. So 24 hours, 72 hours, 168 hours and 192 hours time points were been chosen.

5.3.2 Cultivations with pelagronic acid

Cultivations had been performed in Smit YPD medium at two different temperatures (28°C and 30°C) with six different above-mentioned strains. A period of five days cultivation and fourteen days cultivations had performed like described above in Materials and Methods with 0.3 % pelagronic acid. HPLC were performed to check the utilization of glucose and based on consumption of glucose, three different timeperiod had been chosen.

Table: 6. A five days cultivation with 3g/l pelagronic acid at 30°C.

Substrate	STRAIN					
	Δ MFE4C			Δ MFE+ At red+ Nt P450		
	47 h	91 h	164 h	47 h	91 h	164 h
Nonanoic acid(g/l)	1,75	1,54	0	1,37	0,43	0
Azelaic acid(mg/l)	0	0	0,68	0	12,05	78,29

Table: 6 Comparison of two different *Yarrowia lipolytica* strains (Δ MFE4C and Δ MFE+ *Nicotiana Tabacum* p450hydroxylase + *Arabidopsis thaliana* P450 reductase complex) with 0.3 % pelagronic acid as a substrate and the amount of diacids produced at 30°C. Samples were analysed in GC-MS with three different time periods (47hours, 91hours and 164hours).

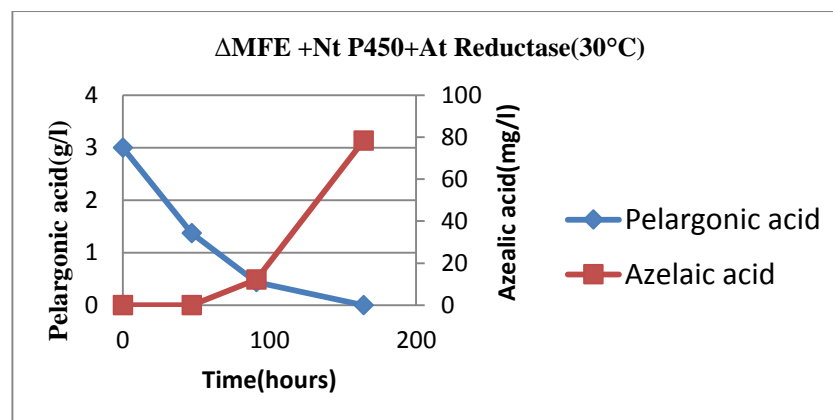


Figure 28. Cultivations of *Yarrowia lipolytica* Δ MFE + Nt P450 + At Reductase (30°C). The substrate (3g/l) were added after 24h to culture grown in Smit-YPD medium. Figure shows the utilization of substrate (diamonds) and the amount of azelaic acid (squares) produced at three different time points.

The table below contains results of GC-MS after 14days cultivations at 28°C . The substrate used was 3g/l of pelagronic acid which were added after 24h cultivation. A comparison of three different time points 168h, 216h and 312h had been chosen based on

HPLC results. The comparison with six different mutants of *Yarrowia lipolytica* were considered and it shows the Δ MFE+ Nt Rd+P450-1clone utilized substrate completely after 216hour and produced 64,08mg/l of diacids after 312hour cultivation. Whereas the other strains (wild type, only Δ MFE 1 and Δ MFE 12 strain) didn't produce any diacids. The cytochrome p450 overexpression complex in mutant strains resulted in diacid production of different amounts.

Table: 7. A Fourteen Day cultivation with 3g/l pelagronic acid at 28°C

Strains	168h		216h		312h	
	Non-anoic acid	Azelai c acid	Nona-noic acid	Azelaic acid	Nonanoic acid	Azelaic acid
Yarrowia wild type	0	0	0	0	0	0
Δ MFE 1	0,83	0	0,75	0	0	0
Δ MFE 12	0	0	0	0	0	0
Δ MFE+ Nt Rd+P450-16(1)	0,01	11,28	0	0	0	9,60
Δ MFE+ Nt Rd+P450-16(2)	0,07	32,01	0	14,11	0	86,32
Δ MFE+ NtRd+P450 clone 1	0,01	32,63	0	47,44	0	64,08
Δ MFE+ NtRd+P450 clone 4	0	16,12	0	52,80	0	0

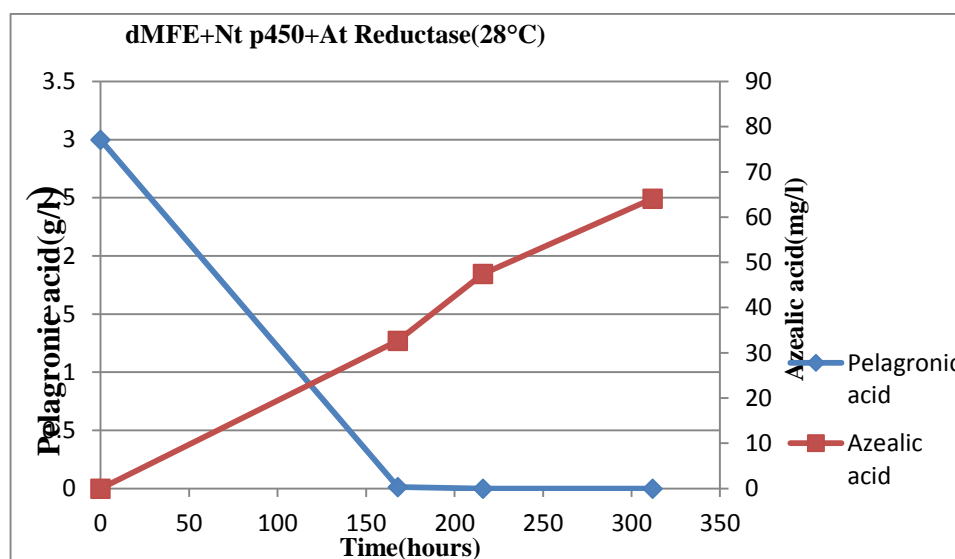


Figure: 29. Cultivations of *Yarrowia lipolytica* Δ MFE +Nt P450+At Reductase clone 1 (28°C). The substrate (3g/l) was added after 24h to culture grown in YPD medium. Figure shows the utilization of substrate (diamonds) and the amount of Azelaic acid (squares) produced at three different time points.

The Table 9 explains the 14-day cultivations at 30°C with substrate 3g/l of pelagronic acid, which were added after 24h cultivation. Six different time points had been chosen (72h, 120h, 168h, 216h, 240h and 288h) based on glucose utilization according to HPLC results. According to this table at 30°C a Δ MFE+ NtRd+P450 clone 1 utilized complete substrate after 168hour and produced 70,747mg/l of C9 diacids at 216hour

cultivation. There had a drop in diacids production at 240hour whereas at 30° Δ MFE+ NtRd+P450 clone 16 was been able to produce 64,733mg/l.

Table 8. 14 Day cultivation with 3g/l pelagronic acid at 30°C

Strains	Pelagronic acid(g/l)					
	72h	120h	168h	216h	240h	288h
Yarrowia wild type	0	0	0	0	0	0
Δ MFE 1	1,81	1,53	0,31	0	0	0
Δ MFE 12	1,43	1,80	0	0	0	0
Δ MFE+ Nt Rd+P450-16(1)	1,49	0,92	0	0	0	0
Δ MFE+ Nt Rd+P450-16(2)	1,57	0,619	0	0	0	0
Δ MFE+ NtRd+P450 clone 1	1,42	0,88	0	0	0	0
Δ MFE+ NtRd+P450 clone 4	1,58	1,12	0	0	0	0

Table 9. A 14 Day cultivation with 3g/l Azelaic acid at 30°C

Strains	Azelaic acid(mg/l)					
	72h	120h	168h	216h	240h	288h
Yarrowia wild type	1,36	0	0	0	0	1,66
Δ MFE 1	0,34	2,33	6,47	0	4,57	0
Δ MFE 12	8,90	0	0	0	0	0
Δ MFE+ Nt Rd+P450-16(1)	9,05	25,75	37,57	17,49	64,73	21,08
Δ MFE+ Nt Rd+P450-16(2)	0	36,03	59,77	47,37	45,72	36,10
Δ MFE+ NtRd+P450 clone 1	12,48	27,54	52,49	70,74	68,16	11,88
Δ MFE+ NtRd+P450 clone 4	0	21,31	30,03	16,47	16,89	0

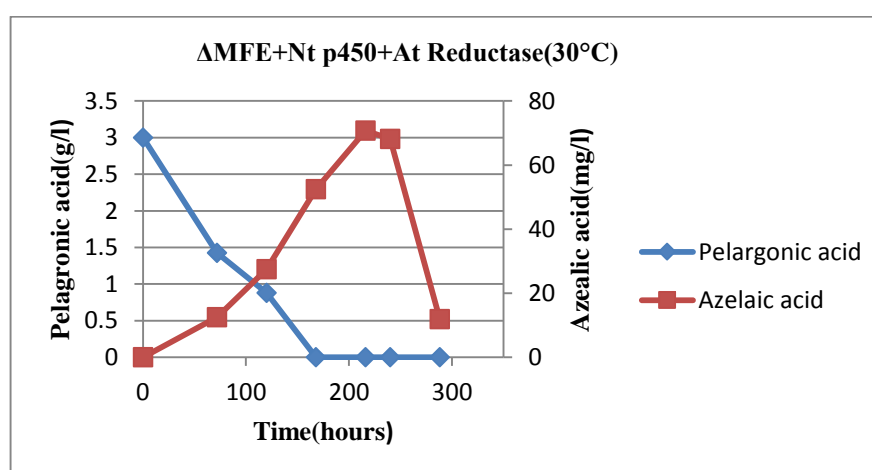


Figure: 30. The production of Azelaic acid (mg/l) by using pelagronic acid (g/l) as a substrate with Δ MFE +Nt P450+At Reductase strain. Comparisons of diacid with six different time points were taken. An Azelaic acid production at 30°C were been plotted. Pelagronic acid (g/l) (diamonds) Versus Azelaic acid (mg/l) (squares) was drawn at 30°C.

5.3.3 Cultivations with oleic acid (C18:1)

Cultivations had been performed with seven different mutants of *Yarrowia lipolytica* in YPD medium at two different temperatures 28°C and 30°C. A period of five days cultivations and fourteen days cultivations had been performed with 10g/l oleic acid. Substrate were been added after 24hours to culture and cultivated with 250rpm shaking. HPLC were performed to check the utilization of glucose and based on consumption of glucose at three different time points had been chosen.

Table 10. Comparison of two different strains of *Yarrowia lipolytica* Δ MFE4C and Δ MFE+ *Nicotiana Tabacum* p450+ *At Reductase* clone 16 with 1 % oleic acid as a substrate. Samples were analysed in GC-MS with three different time points 47hours, 91hours and 164hours.

Substrate	STRAIN					
	Δ MFE4C			Δ MFE+ <i>At red</i> + <i>Nt P450</i>		
	47 h	91 h	164 h	47 h	91 h	164 h
Oleic acid	3,40	1,34	0,18	4,77	0,07	0,06
Di acid	0	0	0	0	0	0

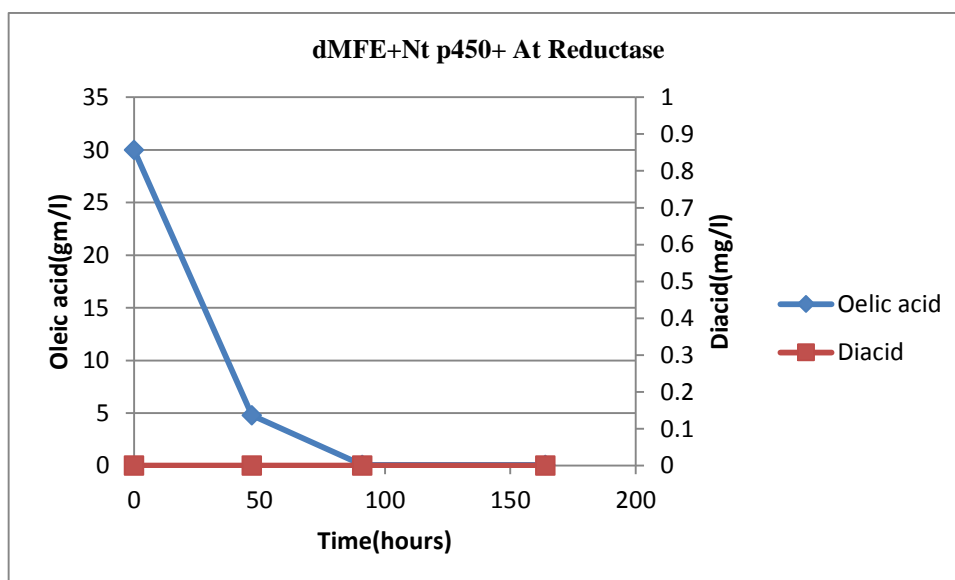


Figure: 31. Comparison of two different strains of *Yarrowia lipolytica* Δ MFE4C and Δ MFE+ *Nicotiana Tabacum* p450+ *At Reductase* clone 16 with 1 % oleic acid as a substrate. Samples were analysed in GC-MS with three different time points 47hours, 91hours and 164hours. Both the mutants utilized substrate periodically but there is no diacids production with both the mutants. Figure shows the utilization of substrate (oleic acid) (diamonds) and the amount of diacid (squares) produced at three different time points

The below two tables were taken from GC-MS results where 14days cultivations of 1% oleic acid were performed at two different temperatures 28°C and 30°C at

250rpm. In this case mutants Δ MFE+ Nt Rd+P450-16(1), Δ MFE+ Nt Rd+P450-16(2) and Δ MFE+ Nt Rd+P450-clone 1 utilized glucose completely based on HPLC results different time periods were been chosen 24hours,72hours,120hours and 192hours at 28°C. At 30°C the complete utilization of glucose had been seen in 24hours, 72hours, 168 hours and 192hours. The same mutants were able to utilize substrate(oleic acid) completely at both the temperature's but there was no diacids production in any of the mutants.

Table : 11. Fourteen-Day cultivation with 30g/l oleic acid at 28°C.

Strains	Oleic acid(mg/l)(28°C)			
	24h	72h	120h	192h
Yarrowia wild type	0,88	0	0	0
Δ MFE 1	3,85	0.09	0,002	0
Δ MFE 12	0,44	0,07	0	0
Δ MFE+ Nt Rd+P450-16(1)	0,02	0	0	0
Δ MFE+ Nt Rd+P450-16(2)	0	0	0	0
Δ MFE+ NtRd+P450 clone 1	0	0	0	0
Δ MFE+ NtRd+P450 clone 4	0,65	0	0	0

In 30°C temperature, no mutant of *Yarrowia lipolytica* used glucose completely at 24hours and no complete utilization of substrate. But at 72hour point there was been a complete utilization of oleic acid by all clones of Δ MFE+ Nt Rd+P450 complex. This shows that there is a complete utilization of substrate by all strains at 168hours but there is no diacids formation in mutants.

Table: 12. Fourteen Day cultivation with 30g/l oleic acid at 30°C.

Strains	Oleic acid(mg/l)(30°C)			
	24h	72h	168h	192h
Yarrowia wild type	0,85	0,19	0	0
Δ MFE 1	2,83	0,07	0	0
Δ MFE 12	2,11	0,00	0	0
Δ MFE+ Nt Rd+P450-16(1)	2,75	0	0	0
Δ MFE+ Nt Rd+P450-16(2)	2,78	0	0	0
Δ MFE+ NtRd+P450 clone 1	3,06	0	0	0
Δ MFE+ NtRd+P450 clone 4	2,74	0	0	0

5.4 *Pichia guilliermondii*

The below table shows, the plasmids constructed in this work:

Table: 13. The Table includes Bacterial strains:

Name	Plasmid name	Backbone	Cloning sites	selection	E.coli strain
pBC8	pBluescript_Pg_ARS	pBluescript SK-	ApaI	Ampicillin	DH5α
pBC6	pBlue_Pg_Cre	pBluescript SK-	EcoRI/SacI	Ampicillin	DH5α
pBC50	pBlue_ARS_Hph	pBluescript SK-	Gibson assembly	Ampicillin	DH5α

5.4.1 ARS plasmid (pBC8)

ARS sequence were been amplified by using BC3/ BC4 oligo pair from *Pichia guilliermondii* strain. These primers were designed based on published ARS sequence available with *ApaI* restriction sites. Genomic DNA of *Pichia guilliermondii*C-72064 strain was used to amplify ARS sequence. A gel check had been performed to confirm the PCR product. All PCR fragments contained the right size. The detected band size is 870 base pairs as expected. These were compared with 1kb Gene Ruler DNA ladder. Thus the construction of plasmids were successful and confirmed by sequencing.

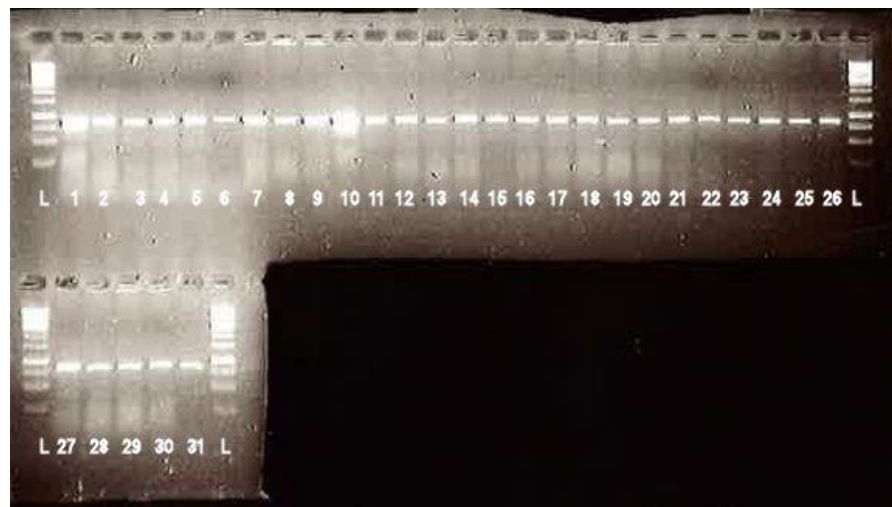


Figure 32. The above gel picture explains the ARS sequence confirmation and Ladder was on both the sides of a gel. Lane1-Lane31 contains amplified products of *Pichia guilliermondii* and expected band is 870bp.

5.4.2 CRE plasmid (pBC6)

CRE cassettes had been cloned into pBluescript (SK-). The Colony PCR check were performed to the selected colonies with M13Forward and BC67primers. M13 forward was from the pBluescript and BC67 Pg_Tef1_promoter_rev1 was from the TEF promoter in Cre cassettes. Bands been clearly obtained with 400bp as expected and absence of bands

in few lanes showed there was no Cre cassette in plasmid and selected plasmids were confirmed by sequencing.



Figure 33. A colony PCR performed gel picture was seen and two L-ladders were placed on both the corners of the gel. Lane 1-Lane 26 has CRE colonies. A clear band of 400bp was obtained. Few lanes had just smear and there were no clear bands in few lanes.

5.4.3 HPH + CRE plasmid

Hph cassettes from pMA plasmid were tried to clone into pBC6 (pBluescript+Cre) but they were not able to be cloned. Below gel picture shows the restriction digestion from stets and digestion had been performed by *EcoRI* and *XhoI*. Expected bands were 5220bp and 2351bp. But there were no expected bands seen in any of colonies. This Restriction digestion was repeated more than twelve times and screened approximately 164 colonies. But couldn't find the correct clone.



Figure 34. The upper gel and lower gel L indicates ladder on last rows of gel. Lane 1-Lane 32 were colonies prepared stets and (pBluescript+Cre+Hph) performed Restriction digestion. The expected bands were 5220bp and 2351bp.

5.5 Gibson assembly

The Gibson assembly had been performed for *Pichia guilliermondii* and the method was performed according to the protocol given by *New England's*. BioLabs Inc.. The below gel shows colony PCR in order to check the correct plasmids. It had been checked by oligo

pair BC67 and BC76. BC67 was from Tef1 promoter from Cre cassette(reverse) and BC76- Hph marker (forward). The expected band were 750bp and it showed in lane 2.



Figure 35. In this gel the L indicates ladder. Lane1-Lane4 were transformants that were from (pBluescript+Cre+Hph+ARS). The expected bands were been 750bp.

Another colony PCR check was performed to the same above transformants but with different oligo pair: BC73 and BC74, BC73 sequence is from Hph marker Eno₂ promoter and BC74 was from Hph cassette. The expected bands were as 750bp but since it is, a colony PCR all bands were not so clear. However, the plasmids were been given for sequencing.



Figure 36. In this gel the L indicates ladder. Lane1-Lane4 were transformants that were from (pBluescript+Cre+Hph+ARS). The expected bands were 750bp.

In order to check the correct transformants, all the above transformants had been given for sequencing to GATC biotech (Germany). Sequencing results shows there were pBluescript(SK-)+ARS+Hph inserts but there was no Cre recombinase cassette. Though the PCR product shows the fragment by sequencing results it was confirmed that there were no Cre cassette.

5.5.1 ARS sequence of *Pichia guilliermondii*

The *Pichia guilliermondii* has only one published Autonomously Replicating Sequence and it had been compared with pBC8 sequence. Clustal-W were used to perform this comparison.

- 1- *Pichia* - This represents published sequence of ARS *Pichia guilliermondii* strain.
- 2- *Pichia*_78ARS- This represents pBC8 sequence from this work.

The results indicate an alignment score of 99.76. This indicates the sequence in pBC8 clone was almost similar to the published sequence.

Table: 14 .Scores table of *ARS Pichia guilliermondii*

Scores Table

[View Output File](#)

SeqA	Name	Length	SeqB	Name	Length	Score
1	1_PICHIA	827	2	78_PICHIA	1293	99.76

Comparison sequence

1_Pichia	-----GTTGACCAACAAT	13
78_Pichia	ATACGACTCACTATAGGGCGAATTGGGTACCGGGCCCGTTGACCAACAAT	50

1_Pichia	CCCGATAAAATTGCTGCAGTTGAGGGAAGAAACAGAGAGGTCAAGGTAGT	63
78_Pichia	CCCGATAAAATTGCTGCAGTTGAGGGAAGAAACAGAGAGGTCAAGGTAGT	100

1_Pichia	GGAACGGGTGCCTATGGTGCCGTTGGCATGGAGAAGTGAGAATGGAATCA	113
78_Pichia	GGAACGGGTGCCTATGGTGCCGTTGGCATGGAGAAGTGAGAATGGAATCA	150

1_Pichia	AGTCAAAAGAGATAGAGGGCTACTTGAGTGCTAAGATTGAAAAGGATGGGG	163
78_Pichia	AGTCAAAAGAGATAGAGGGCTACTTGAGTGCTAAGATTGAAAAGGATGGGG	200

1_Pichia	CACTTGCTTGAAAAGCCACTCAAGATATGATAGAAGAGATGAAGTTAAGG	213
78_Pichia	CACTTGCTTGAAAAGCCACTCAAGATATGATAGAAGAGATGAAGTTAAGG	250

1_Pichia	ACTTAAGAAATAAATGATGAATTAAATGACGCAAAATGTCACTACTCGATT	263
78_Pichia	ACTTAAGAAATAAATGATGAATTAAATGACGCAAAATGTCACTACTCGATT	300

1_Pichia	AGAGAAATAGCTATAATGAAGAAATTTTGCATTTTCGCAAAATTTAAGATAA	313
78_Pichia	AGAGAAATAGCTATAATGAAGAAATTTTGCATTTTCGCAAAATTTAAGATAA	350

1_Pichia	ATGCAAAAATGCAAAATTACGAAATATGCATATGATACAAGACAAGAAAA	363
78_Pichia	ATGCAAAAATGCAAAATTACGAAATATGCATATGATACAAGACAAGAAAA	400

1_Pichia	GACTACCAAAAGTCTCTCGAGAAGAATACTGGGTAACTTCATCTCTTGA	413
78_Pichia	GACTACTAAAAGTCTCTCGAGAAGAATACTGGGTAACTTCATCTCTTGA	450

1_Pichia	TTATGCACTGGGGCTATTCATATGCAGATTTCGCACGCCGAGGTGCAGCGT	463
78_Pichia	TTATGCACTGGGGCTATTCATATGCAGATTTCGCACGCCGAGGTGCAGCGT	500

1_Pichia	TTAGGCGCGGCTCAACGGAAGCCAAACGGCCGCCACAAATTGTCCGGAAAAG	513
78_Pichia	TTAGGCGCGGCTCAACGGAAGCCAAACGGCCGCCACAAATTGTCCGGAAAAG	550

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1_Pichia   TCGCCGAAACTGATCCACTGGTA----- 536
78_Pichia  TCGCCGAAACTGATCCACTGGTAAACGGAAAGCCAACGGCCGCCACAAATT 600
          *****

1_Pichia   -----CCACAGCCCCATAAGAA 553
78_Pichia  GTCCGGAAGTCGCCGAAACTGATCCACTGGTACCACAGCCCCATAAGAA 650
          *****

1_Pichia   CCCCTTTAATATTAAAAACCGTTCTTCAGCCACTTTTGATCACATTGTT 603
78_Pichia  CCCCTTTAATATTAAAAACCGTTCTTCAGCCACTTTTGATCACATTGTT 700
          *****

1_Pichia   TGCAGCCGCCCGTTGCTGCCATCCAAACACCACGCGTCCCCCGCACCTTT 653
78_Pichia  TGCAGCCGCCCGTTGCTGCCATCCAAACACCACGCGTCCCCCGCACCTTT 750
          *****

1_Pichia   TTACGGTGCCCACTGCATTGGAATTTGCATAAAACAGCCTCACGAAGTGG 703
78_Pichia  TTACGGTGCCCACTGCATTGGAATTTGCATAAAACAGCCTCACGAAGTGG 800
          *****

1_Pichia   ATTAATTTTATAGAGCACTCAAGTCATCATGCTGCAATCTCTGCATCATGA 753
78_Pichia  ATTAATTTTATAGAGCACTCAAGTCATCATGCTGCAATCTCTGCATCATGA 850
          *****

1_Pichia   AATGACTCCCGTTGATACAGGGAAGTCAGACCGCAAGCGGCGAAGAGTCA 803
78_Pichia  AATGACTCCCGTTGATACAGGGAAGTCAGACCGCAAGCGGCGAAGAGTCA 900
          *****

1_Pichia   CAAGAGCGTGTGATGTGTGTGCGAC----- 827
78_Pichia  CAAGAGCGTGTGATGTGTGTGCGACGGGCCCCC 932
          *****

```

6. DISCUSSION

Cre recombinase lox-P system containing plasmids had constructed for *Yarrowia lipolytica* and *Pichia guilliermondii* yeast strains. Autonomously replicating sequences (ARS), a Cre recombinase and Hygromycin marker genes with specific promoters and terminators had been used to construct the plasmid.

6.1 ARS

Yarrowia lipolytica ARS with 1300bp sequence had been amplified from C-00365 and cloned into pBluescript (SK-). The obtained sequence from thesis work was compared with published sequences by using Clustal-W. ARS18 and ARS68 were two published sequences that were available for *Yarrowia lipolytica*. After multiple sequence alignment comparison it is clearly observed that the available sequence was similar to ARS18 with 99.69 similarity. There was a less alignment similarity with ARS68 only 64.56 score similarity was seen. From results, it had clearly proved that ARS from thesis work was more similar to ARS18 than ARS68.

Pichia guilliermondii ARS sequence with 827bp was amplified from C-72064 genomic DNA. A comparison of multiple sequence alignment were performed with pBC8 sequence and available published sequence a similarity of 99.76 were obtained.

6.2 CRE cassettes

Cre cassette contains in addition to codon optimised recombinase genes elongation factor 1-alpha (TEF1) promoter and Triose phosphate dehydrogenase (Tdh3) terminator. Cre cassettes from pMA plasmids had been cloned into pBluescript by *EcoRI* and *SacI* sites. *Table 4* and *Table 13* clearly shows the plasmid names and with cloning restriction sites. Cre cassettes construction of *Yarrowia lipolytica* and *Pichia guilliermondii* was confirmed by Colony PCR and by sequencing.

6.3 HPH cassettes

Hph cassette contains Hph marker gene encoding for *Y. lipolytica* with two LoxP sites at the corners of Enolase 2 (ENO2) promoter and phosphoglyceratekinase (PGK) terminator.

P. guilliermondii (Pg_loxp_EnO2_promoter_loxp_Pgk1_terminator) same as like *Y. lipolytica* with same promoter and terminator from pMA plasmids had been cloned into Cre cassettes + pBluescript of *Yarrowia lipolytica* and Cre cassettes + pBluescript of *Pichia guilliermondii* by using *EcoRV* and *XhoI*. In this case *Yarrowia lipolytica* Hph

marker cassettes were able to be cloned into Cre cassettes + pBluescript but where as for *Pichia guilliermondii* unfortunately couldn't be cloned into Cre cassettes + pBluescript. Therefore, Gibson assembly was performed to alter this method.

6.4 Gibson assembly

Pichia guilliermondii with ARS sequence and Cre recombinase by traditional method was successfully constructed but unfortunately Hygromycin marker gene were not able to insert into pBC6 clone. Gibson assembly was a new technique where combination of amplified inserts with adjacent insert flanks were used to construct complete plasmid vector. NEB builder were been used to design primers, inserts were amplified by PCR and after Gibson assembly transformed into DH5alpha Bacterial strains according to protocol mentioned in New England Inc., Unfortunately, only pBC50(pBluescript+ARS+Hph) clone was constructed. In this technique Cre recombinase had been missing to get complete *Pichia guilliermondii* plasmid vector for transformation into *MFE2* deleted strains.

6.5 Confirmation of the Nat marker loop out transformant

A complete plasmid vector of *Yarrowia lipolytica*(pBC27) had tested by transforming into *Yarrowia lipolytica*MFE2 blocked strain by using LiAc method. By seeing Figure 24, it indicates a successful loop out of nourseothricin(400µg/ml) marker from Genomic DNA and Cre recombinase plasmid had also looped out.

6.6 Confirmation of the *Nicotiana Tabacum* P-450 hydroxylase and *Arabidopsis thaliana* P450 reductase.

To enhance the cytochrome P450 complex in ω -oxidation *Nicotiana Tabacum* P450 hydroxylase and *Arabidopsis thaliana* P450 reductase were been expressed in the *Yarrowia lipolytica* MFE2 deleted strain successfully. This was confirmed by the diacid production in performed cultivations.

6.7 Cultivations

Cultivations were been performed in YPD medium at two different temperatures: 28°C and 30°C with six different strains of *Yarrowia lipolytica*. A period of five days cultivation and fourteen days cultivation were performed with 0.3 % pelagronic acid and with 1 % Oleic acid.

At 28°C of five day cultivations with pelagronic acid 47h, 91h and 164h according to the table 6 and figure 28 says as the substrate was consumed the Δ MFE+ At red+ Nt P450-16 clone had been able to produce 78,29mg/l of Azelaic acid. Whereas Smit et.al, 2005 observed the production of dioic acids in *Yarrowia lipolytica*, by deleting six POX genes, they were been able to accumulate only 18 mg ml⁻¹ dodecanedioic acid by using

12-hydroxydodecanoic acid as a substrate. A less amount of Azelaic acids was produced by Δ MFE+ At red+ Nt P450 clone1 and Δ MFE+ At red+ Nt P450-4 that is 64,08mg/l and 53,80mg/l. There was no diacid production in wild type of *Yarrowia lipolytica* and Δ MFE 1, Δ MFE12. At 30° Ccultivation's on 216 hour the azelaic acid produced by Δ MFE+ At red+ Nt P450 clone1 and Δ MFE+ At red+ Nt P450-16 was only70mg/l and 47,376mg/l. So, by this we can conclude that a good amount of azealic acid was produced at 28° C.

Oleic acid cultivations were also performed at both the 28° C and 30° C temperatures. According to table 10 and figure 31it clearly showed that there was a complete utilization of oleic acid (substrate) and unfortunately there were no production of diacid at any time point. Where as Fabritius and his colleagues (1996) showed that there was formation of less than 5g/l of 3-hydroxyhexadecenedioic acid,1,6-hexanedioic acid, ω -hydroxy octadecenoic acid and 19.4g/l of hydroxydioic acid at 233hour by using oleic acid as a sole carbon source in fed-batch fermentation in *candida tropicalis* mutants.

6.8 Comparision with *Candia tropicalis*

Hara et.al, 2001 studied Repression of POX 4 gene in *Candida tropicalis* (strain M1210A3) which encodes fatty acyl-CoA oxidase activity and the strain had been able to produce 17.6 g/l of Carbon₁₃ diacid when these were compared with absence of plasmid it was able to produce 14.4g/l of C₁₃ dicarboxylic acid. In a study with β -oxidation blocked completely and by over expressing of cytochrome P450 ALK1 gene of *Candida tropicalis* it was able to produce 150g/l of dioic acid by using dodecane and tridecane as a substrate at 92hours (H5343 strain) (Picataggio et.al, 1992).

According to Huf, 2011 USA, Germany and Japan have done most of their research on *Candida tropicalis* and developed strain for producing high yield production of dioic acids but they couldnot receive up to the mark. In Europe, they considered *Candida tropicalis* as a pathogenic industrial microorganism and need high level of standards to use in industrial purposes. However, *Yarrowia lipolytica* and *S. cerevisiae* had been studied but they couldnot reach the levels of *C.tropicalis* in diacid production.

Multifunctional enzymes perform the hydration and dehydrogenation step of β -oxidation pathway in higher eukaryotes. There were two Multi functional enzymes in mammals but in yeast only one *MFE2* is present and there is no evidence until now for the presence of isozymes in fungal species or yeast(Bogaert, et.al, 2009). So, MFE2 were targeted in these studies where as there are six POX genes are present so deleting one single MFE 2 gene is easier than the other genes. Nevertheless, in recent studies of Vorapreedaan interesting result were out by comparing oleaginous yeast and non-oleaginous yeast genomes says in oleaginous microbes in addition to peroxisomal β -oxidation, fatty acid degradtaion also occurs in mitochondria. There was an evidence clearly that

mitochondrion orthologous targeted proteins which were involved in β -oxidation. *Yarrowia lipolytica* possesses an incomplete set of mitochondrial β -oxidation enzymes acyl CoA dehydrogenase, 2-enoyl CoA hydratase and 3-ketoacyl CoA thiolase. By this evidence it's concluded that fatty acid degradation also occurs in mitochondria. Mitochondrial β -oxidation pathway was a type I that involves in breaking down of medium and short chain fatty acids. (Voraoreeda, T., et.al, 2012) However, our results shows small amounts of C9 diacid production which was lower than compared to *Candida tropicalis*. From industrial point of view, the amount produced were very less but on the other hand the application of C9 diacid is high and production was in sustainable way from genetically engineered micro organisms. This was not as profitable to industry they need to produce in larger amounts. However, the results of this work show more bottleneck and other limiting factors in engineering Beta Oxidation pathway. By this metabolic engineering work of this thesis proved that there were production of dicarboxylic acids from alkanes and fatty acid in *Yarrowia lipolytica*. But essential modifications would be needed to further study and develop this strain for industrial purpose as it was considered as non-pathogenic.

7. CONCLUSIONS

Long chain DCA production was demonstrated with yeast *Yarrowia lipolytica*. Cre recombinase plasmid had been constructed successfully. A Successful loop out of nourseothricin (400µg/ml) marker from genomic DNA and Cre recombinase plasmid from *MFE2* deleted *Yarrowia lipolytica* strain could be observed. Overexpressing the first enzymes of ω -oxidation enzyme Nicotiana *Tabacum* P 450 hydroxylase and *Arabidopsis thaliana* P450 reductase (P450 oxidoreductase) in the *Yarrowia lipolytica* *MFE2* deleted strains were successful. Cultivations with pelagronic acid were been successful and 78,29mg/l of C9 diacids were produced. Whereas with Oleic acid only substrate consumption was seen: There was no diacid production.

Pichia guilliermondii was constructed by two different methods a traditional method and Gibson assembly. A plasmid with two genes of interests was been able to construct by Gibson assembly. PBluescript with ARS and Hph were successful by Gibson assembly. Whereas in traditional method pBluescript+ARS and pBluescript+Cre was constructed .The work on *Pichia guilliermondii* can be carried further by constructing complete plasmid vector.

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